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Studies on the antigenicity of streptolysin

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To Paula

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INTRODUCTION

Streptolysin S, a streptococcal toxin of current interest, consists of a polypeptide attached to a polyribonucleotide (40)(41). The active component of the complex appears to be polypeptide, and the polyribonucleotide functions as a carrier (20).

Possible Role of Streptolysin S in the Pathogenesis of Rheumatic Fever: Rheumatic fever is a non-suppurative sequel of streptococcal infections, but its pathogenesis remains obscure. Auto-antibodies to heart tissue have been demonstrated in this disease (38)(28), but whether they play a pathogenetic role remains questionable. Most auto-antibodies in other clinical and experimental conditions appear to be innocuous and some may even be protective (46)(76).

The natural history of rheumatic fever, with its multiple recurrences without shortening of the latent period, (51) is unlike the experimental models of allergic diseases and could well be explained by a non-antigenic toxin of group A streptococci. Such is the oxygen-stable streptococcal hemolysin, or streptolysin S. A potent hemolytic and lysosome-disrupting agent (82), this toxin has some added features which further

increase its likelihood of being the agent of damage in rheumatic fever: (I) it can be produced by non-dividing cells (1), such as one would expect to find in the convalescent stage of streptococcal infections, during the latent period of rheumatic fever; and (II) its inhibitor, normally present in the serum of man and other animals, is decreased in patients with active rheumatic fever (60)(79). Another intriguing observation is the mitosis-stimulating effect of streptolysin S on the peripheral lymphocytes of normal subjects and of patients with diseases other than rheumatic fever, but not of patients with rheumatic fever (30). It is therefore believed that this study on the immunogenicity of streptolysin S may provide information relevant to the pathogenesis of rheumatic fever. If a means were found to immunize experimental animals against streptolysin S, it would be of interest to adapt such means to man, and test the hypothesis that immunization may prevent rheumatic fever.

At the outset it is important to note that streptolysin S is presently considered to be non-antigenic. More specifically, one may state that heretofore streptolysin S (as it is prepared in the laboratory) has been shown to be non-antigenic under certain well-defined conditions (to be discussed in the next subdivision).

However, a categorical statement cannot justifiably be made as to the antigenicity of streptolysin S because many alternative methods of demonstrating its antigenicity or lack of antigenicity remain to be explored.

The possibility of making streptolysin S antigenic by utilizing it as a hapten and coupling it to a protein moiety which would serve as a carrier has not been tested. Such an approach will be used in the present study.

Antigenicity of Streptococcal Hemolysins: The study of streptococcal hemolysins was greatly clarified by Todd (78), who first clearly differentiated streptolysin S, a serum-soluble, oxygen-stable hemolysin, from streptolysin O, which is readily formed in serum-free media and is inactivated by oxygen. It has been shown by him (77), and by many workers since, that streptolysin O is a powerful antigen, and that infection with strains of streptococcus which produce this hemolysin is generally followed by a sharp rise in the antistreptolysin O content of the serum.

In 1939, Todd, Coburn, and Hill published a study of antistreptolysin S and O titers in normal adults, non-rheumatic children with, or recovering from, hemolytic streptococcal infections, rheumatic children suffering from similar infections, but with no rheumatic activity,

and rheumatic children in whom streptococcal infection was followed by an acute attack of rheumatic fever. The variations in the antistreptolysin S titer were not large. However, from a statistical analysis of their data, these authors concluded that in response to infection with hemolytic streptococci there is a rise in the antistreptolysin S titer considerably above the normal level in the sera of all groups studied. Those rheumatic children who developed clinical signs of rheumatic activity were the only exception. In the sera of these children the antistreptolysin S titer, although often above normal, tended to remain relatively low. Sera taken during the period of acute rheumatic fever showed lower antistreptolysin S titers than sera taken from the same patients during periods of rheumatic inactivity. Furthermore, during rheumatic attacks the antistreptolysin S titers tended to be lowest when the clinical symptoms were most pronounced. Todd et al. regarded the antistreptolysin S titer of human sera as due to a specific antibody. The fall in titer which occurred during rheumatic activity they considered as evidence of an abnormal antibody response (79).

Humphrey (33) conducted a study on sera taken from patients with streptococcal infections, rheumatic fever, and rheumatoid arthritis in various stages or their disease

as well as in normal controls. Nearly all sera taken from sick persons had lower antistreptolysin S titers than those from normal controls. The highest antistreptolysin S titers were found in normal adults and in late convalescent cases of rheumatic fever. In the acute phases of streptococcal infections, rheumatic fever, and rheumatoid arthritis and during the early stages of convalescence, the antistreptolysin S titers were comparatively low.

Stollerman and Bernheimer's study in 1950 (60) confirmed the phenomenon of decreased antistreptolysin S titers in the sera of patients with clinically active acute rheumatic fever first noted by Todd et al. (79). It was also apparent that the fall in titer was not a decrease in antibody, but more likely reflected a metabolic disturbance which resulted in a diminution of a normal component of serum. That the fall in antistreptolysin S is not specific for rheumatic fever was demonstrated by the finding of low titers in some cases of diseases unrelated to streptococcal infection (60). The failure of any patient in this study to show a rise in the serum titer against streptolysin S following proved streptococcal infection, or during the course of rheumatic fever, when streptococcal antibodies had been demonstrated at very high levels, is evidence that streptolysin

S is not antigenic in natural streptococcal infections in man. On the basis of these facts, in conjunction with the constancy and narrow range of antistreptolysin S titer in normal human sera, Stollerman and Bernheimer suggested that the inhibition of streptolysin S by human serum is due to a normal component of serum rather than to a specific antibody. They also suggested that the term "antistreptolysin S" be abandoned since this denotes an antibody, and they substituted the noncommittal expression "streptolysin S inhibitor" (SSI).

Todd reported that streptolysin S is not antigenic when injected by itself. Four rabbits were injected subcutaneously and intravenously with approximately 36,000 50% hemolytic units of streptolysin S in 16 doses, without eliciting an antistreptolysin S rise. He, however, initially claimed that by prolonged courses of intravenous injections of living Group A streptococci into rabbits it was possible to obtain antisera to streptolysin S. (One rabbit developed an antistreptolysin S titer of 400 one week after a repeat I.V. injection with living streptococci; other rabbits similarly treated developed no rises or smaller rises--to less than 100 units. About half the rabbits died during the course of the immunization with generalized streptococcal infections) (78) (27).

Humphrey (33) repeated these immunization efforts by giving rabbits intravenous injections of streptococci. The first two injections consisted of killed organisms, but all later injections were of living streptococci. Injections were given two or three times weekly, with rest periods of 20 days or more in between courses. The total volume of packed living organisms injected varied from 0.6 to 1.0 ml., and of culture fluid from 12 to 20 ml. A horse was also injected with five intramuscular doses of formolized culture filtrate from streptococci grown in yeast nucleic acid broth. These five injections were followed by a total of nearly three liters of untreated filtrate, in 26 doses over a period of 15 weeks. No evidence was obtained of an increase in antistreptolysin S titer in either the rabbits or the horse.

Stollerman, Bernheimer, and McLeod (61) injected one group of rabbits with one mgm. of streptolysin S subcutaneously twice weekly for two weeks followed by a rest period of two weeks. Twelve injections were given over a period of three months. A second group of rabbits was injected intravenously twice weekly with 0.8 mgm. of streptolysin S per kg. of body weight at each injection. A total of nine injections was given. A third group of rabbits was injected twice weekly according to the schedule outlined by Todd (78), with increasing doses of whole

culture of living hemolytic streptococci grown in neopeptone meat infusion broth for 18 hours at 37°C. Specific antibodies to streptolysin S did not appear and no increase in inhibitory action was observed in these rabbits.

In 1965 Taranta and Bernheimer attempted immunization of guinea pigs against streptolysin S toxoid (74). The reason for using streptolysin S in a toxoid form was to be able to give a very large dose of the toxin without killing the experimental animal. In this experiment a total of 50 mgm. of streptolysin S was injected into each of two guinea pigs with complete Freund's adjuvant (20 mgm./ml., 10,000 units/mgm.), corresponding to 500,000 hemolytic units before formolization and to 5,000 units after formolization. No neutralizing antibody response was detected. Ouchterlony plates with the same streptolysin S preparation were negative for precipitating antibodies.

Thus, all of the attempts described above to immunize animals against streptolysin S were unsuccessful.

Toxicity of Streptolysin S: Weld (83), (84), Hare (26), and Barnard and Todd (2) studied the toxic effect of serum-extracted hemolysin (serum filtrates of living streptococci possessing hemolytic activity) in the mouse. Toxic manifestations depended on the amount of hemolysin

injected. Following the injection of streptococcal extracts containing 15-30 100% hemolytic units* of hemolysin, death occurred in 24 hours; larger amounts caused death within an hour. All of these animals showed dyspnea, weakness, prostration, and intravascular hemolysis. The animals which survived 90 minutes showed hematuria and considerable enlargement of liver and spleen. At autopsy no significant pathological lesions were observed. In animals which survived longer (2-8 hours) there was hematuria and, in the majority of animals, marked degenerative changes in the tubular epithelium of the kidney. The glomeruli revealed no histological changes. There was also marked necrosis of the lymphoid cells in the follicles of the spleen and small focal necrosis in the liver. In animals surviving more than eight hours there was almost always marked jaundice. Animals which survived for one week showed very little pathologic change in the liver, but renal hemorrhages were found. Rabbits showed similar changes to those in mice although in smaller degree. Guinea pigs were more resistant to the effect of hemolysin, no intravascular hemolysis being observed.

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*100% hemolytic unit--That amount of streptolysin S which will cause 100% hemolysis of a .7% suspension of human red cells contained in 2 ml. of phosphate-buffered saline, pH 7, in 30 minutes at 37°C.

Both the hemolytic and pathologic effects described above could be prevented by heating the preparations to 50°C for 60 minutes. Since the preparations of hemolysin used in these studies were not purified, it is impossible to attribute with any degree of certainty the lesions described to streptolysin S specifically. Other streptococcal products also present in the preparations include streptokinase, streptolysin O, and ribonuclease (RNA-se) all of which are susceptible to heating to 50°C for 60 minutes. In view of the more recent findings on the cytopathogenic effects of partially purified albumin-hemolysin, or RNA-hemolysin on mammalian cells and the effect of purified RNA-hemolysin on the basement membrane of the kidney (including inhibition by anti-hemolytic agents) (70), it may be assumed that at least part of the pathological changes induced in the animals described above was caused by streptolysin S.

Todd (78) inoculated two rabbits subcutaneously with serum-extracted hemolysin prepared in normal rabbit serum from a group A strain. Each rabbit received 24.5 cc. of hemolysin--average hemolytic titer approximately 200 100% hemolytic units per cc.--in five doses spread over 12 days. The rabbits were rested for 146 days before a second course of subcutaneous injections was given consisting of 48 cc. of hemolysin in 11 doses spread over 25 days. The first rabbit died during this course while the second remained well.

Two more rabbits were given three courses of serum-extracted hemolysin, the first two subcutaneously and the third intravenously. The rabbits remained in good condition during the period of inoculation and gained weight. Subcutaneous inoculations were followed by local erythema and thickening of the subcutaneous tissues at the site of injection. Intravenous inoculations did not cause any symptoms and there was no evidence of hemoglobinuria. This may be due to the relatively low hemolytic titers of serum-extracted hemolysin.

Todd (78) attempted immunization of 23 rabbits with living cultures of group A hemolytic streptococci. Injection of living cultures was usually preceded by I.V. inoculations of vaccine prepared by heating a broth culture at 56°C for 30 minutes. The animals were then rested for seven days and immunization was begun by I.V. inoculation of 1.0 cc. of supernatant fluid from a broth culture which had been centrifuged for 20 minutes at 3000 r.p.m. Nine of the rabbits died with hemolytic streptococci in their blood, four were killed because of arthritis, and ten survived.

Stollerman et al. (61) injected six rabbits intravenously with streptolysin S in a dose of 0.8 mgm. per kg. of body weight at each injection. The toxin was given twice weekly. Nine injections were given to three

of the animals while three died before the course of injections could be completed.

Ten rabbits were injected twice weekly, according to the schedule outlined by Todd (78), with increasing doses of whole culture of living organisms grown in neopeptone meat infusion broth for 18 hours at 37°C. Two animals died during the first course of injections and one, which developed a large orbital abscess, was sacrificed. The other rabbits lost weight, appeared ill, and in every instance a fall in serum titer of streptolysin S inhibitor occurred. After a period of rest, the seven surviving rabbits were given a second course of injections similar to the first. The second course was tolerated well and the animals did not sicken. None exhibited an elevation of inhibitor levels, but a reversion to the original normal levels took place.

Bernheimer (7) injected 0.10 mgm. streptolysin S (10,000 units/mgm.) intravenously into each of six mice. One died in 3 hours, one in 6½ hours, one in 29 hours, two between 32 and 48 hours, and one survived. 0.05 mgm. of the same streptolysin S preparation was injected intravenously into each of six rabbits. One died in 1 hour and five survived. Five rabbits were injected intracutaneously with 1 mgm. streptolysin S (same preparation as above). Two of these animals died after the first injection

while three survived. Two of the survivors received 18 injections (total of 180,000 units) at the rate of three per week while one received 15 injections (total of 150,000 units) also at the rate of three per week.

Tan, Hackel, and Kaplan (68) observed necrosis of renal tubular epithelial cells in mice harboring streptococci in intraperitoneal diffusion chambers. Tan and Kaplan (69) found that this effect could be produced by the injection of streptolysin S.

Snyder (59) showed that preparations of the RNA-hemolysin were pyrogenic in rabbits and that the pyrogenicity could be abolished by lecithin.

Non-antibody Nature of Serum Streptolysin S Inhibitor: As early as 1949, in the investigation reported by Humphrey (33), antistreptolysin S failed to conform to the accepted definition of an antibody, i.e., a gammaglobulin which combines with an antigen and is produced in response to the introduction of the antigen into the tissues of the organism. Antistreptolysin S was found to occur in the sera of a wide variety of normal animal species, in the absence of evidence of either present or past streptococcal infection; it did not increase on immunization; and the antistreptolysin S activity was not located in the gammaglobulin fraction in the sera of humans,

rabbits, and horses.

The highest degree of streptolysin S inhibition appears in the fractions associated with alpha-1 and beta-1 lipoproteins when serum is fractionated by the cold alcohol method. When serum is fractionated by salting out with ammonium sulfate, the albumin fraction is also associated with streptolysin S inhibition (61). Stollerman *et al.* (61) also found that serum phospholipids, particularly lecithin, play an important part in the inhibition, either as components of lipoprotein which may inhibit streptolysin S, or as stabilizing agents for lipoprotein complexes, or possibly in both capacities. A saline suspension of phospholipids, particularly lecithin, inhibits streptolysin S. A marked reduction of SSI occurs when serum is treated with *Cl. welchii* lecithinase, or with ether or an ether-alcohol mixture. Stollerman *et al.* concluded that SSI in serum appears to be composed of a phospholipoprotein complex and that the phospholipids of serum may play a role in its stabilization as well as in its composition.

Relation of Streptolysin S to Other Streptococcal Hemolysins--Interchangeability of the Carriers of the Hemolytic Moiety: During the past 30 years several oxygen-stable hemolysins have been reported to be

produced in cultures of growing streptococci or in suspensions of resting streptococci. In 1934, Weld (83) showed that a potent hemolysin indistinguishable from the hemolysin later described by Todd (78), could be obtained by shaking washed streptococci with horse or human serum. Todd (78) found that streptolysin S was produced only in media containing whole serum. Okamoto (47) and Hosoya et al. (31)(32) showed that yeast nucleic acid induced the formation of a potent hemolysin in growing streptococcal cultures. Studies by Bernheimer (4)(5), Hosoya and his collaborators (31)(32), and Tanaka, Hayashi, and Maekawa (71) showed that the digestion of yeast RNA with pancreatic ribonuclease markedly increased its hemolysin-inducing capacity.

In 1956 Schwab showed that hemolytic material can be obtained from streptococci subjected to sonic energy for long periods of time (53)(54). The hemolytic material was named intracellular hemolysin. Schwab reported (55) that the intracellular hemolysin is antigenic in rabbits, and thus may be different from streptolysin S. The nature of this material and its possible relationship to the RNA or serum hemolysin remains to be established.

In 1958 Ginsburg and Grossowicz (16) showed that various strains of group A streptococci possess a cell-bound hemolysin. This hemolysin, which may be identical

with that described by Weld (83), could not be released from the cells by sonic disruption or by grinding with glass beads (16), but was released by treatment with serum albumin, Tween-40, -60, -80, or Triton X-205 (16).

The problem of the role played by such diverse materials and substances as serum, albumin, detergents, and RNA in the induction of hemolytic activity from resting streptococci was studied by Ginsburg and Harris (20). They proposed the concept of a carrier-hemolysin complex on the basis of their studies. Their experiments showed transfer of hemolysin from one carrier to another, inactivation of the respective hemolysins by proteolytic degradation of the hemolytic moiety or by hydrolysis or distortion of the carrier molecule, inhibition of the hemolytic activity of all the hemolysins by the same inhibitors, the lack of all these hemolysins in a single streptococcal mutant, and the non-antigenicity of all these hemolysins. These observations lead to the conclusion that a single hemolytic group synthesized by streptococci can be bound by a variety of carriers which have affinity for that active group.

This work also sheds new light on the cell-bound hemolysin of streptococci (83)(16), and its relation to the various forms of extracellular hemolysin described. During the growth of the streptococci in media devoid of

any of the carriers mentioned above, the hemolysin is synthesized and is found closely associated with the bacterial cell. Upon addition of a substance which has greater affinity for this hemolysin than does the streptococcal cell-component involved, the hemolysin is released from the cells to form a complex with this substance. (20).

As to the chemical nature of the carrier essential for the induction or binding of hemolysin, at least three families of compounds may be involved; first, lipids, phospholipids, or fatty acids; second, RNA or RNA core; third, substituted oxyethylene polymers, as Triton X-205 and Tweens. Ginsburg and Harris concluded that these hemolysins represent a single hemolytic group synthesized by streptococci, which can be bound to a number of carriers, the original carrier being a component of the streptococcal cell.

Possible Reasons for the Non-antigenicity of Streptolysin S: Possible reasons for the non-antigenicity of streptolysin S are its small size (molecular weight, 12,000, for the whole lysin or polypeptide-polyribonucleotide complex, as determined by gel filtration) (8) and the looseness of the binding of the polypeptide to the polyribonucleotide as shown by the possibility of transfer of

the hemolytic moiety from one carrier to another (20). According to Koyama (41) the weight ratio of polypeptide to polyribonucleotide of highly purified streptolysin S is 0.3:1. Therefore, if streptolysin S (polypeptide plus polyribonucleotide) has a molecular weight of 12,000 the molecular weight of the polypeptide, the active moiety, is 2,800, and it should consist of approximately 28 amino acid residues.

Antigenicity of Low Molecular Weight Substances: Antigenicity is usually hindered by low molecular weight. However, in recent years several examples of antibody production to low molecular weight substances (even without recourse to a carrier) have been reported in the literature. It is remarkable that Axelrod, Trakatellis, and Hofmann have found that biologically inactive peptides with a chain-length of 16 amino acids (molecular weight less than 2000) are immunogenic (1). Sela, Fuchs, and Arnon showed synthetic polyamino acids, of molecular weight around 4000, to be immunogenic, provided that they were of certain composition and contained certain amino acid sequences (56). Antibodies have been produced against synthetic oxytocin, an octapeptide, by Gilliland and Prout (15). This evidence of antigenicity of low molecular weight substances is in no way contradictory

to the well-established observation that antigenicity increases with increasing molecular weight. Even in the low range of molecular weights the same rule applies: Axelrod et al. found that immunogenicity of small peptides increases with increase in chain length. In their experience a peptide consisting of 20 amino acids was more immunogenic than a peptide consisting of 16 amino acids (1).

Methods to Make Antigenic Substances That are Not Naturally So: At an increasing pace in recent years, antibodies have been produced to substances which are not naturally antigenic by a number of chemical and physicochemical maneuvers. Some of these maneuvers are relevant to the problem at hand.

Historically, Landsteiner's pioneer investigations on the antigenicity of low molecular weight substances are noteworthy. His demonstration (45) that an organic molecule of low molecular weight, e.g., ascorbic acid, functions as a hapten, when it is linked covalently to a protein antigen as a carrier, opened up a new avenue for the study of antibody formation and structure.

Goodfriend, Levine, and Fasman (23) produced antibodies to the low molecular weight polypeptides bradykinin and angiotensin. They used water soluble carbo-

diimides as coupling agents to produce hapten-protein conjugates, e.g., bradykinin-rabbit serum albumin (RSA), in which case bradykinin is the hapten and rabbit serum albumin is the protein.

Butler, Beiser, and Erlanger (9) and Tannenbaum and Beiser (75) showed that purine and pyrimidine bases are haptens when coupled to a protein such as bovine serum albumin and that antibodies to the haptens react with denatured DNA. In addition Goebel and Avery (22) had demonstrated that pneumococcal polysaccharides, which are non-antigenic in the rabbit by themselves, could act as haptens when coupled to a protein. Plescia, Braun, and Palczuk were motivated by these observations to further immunochemical studies of chemically defined DNA-protein complexes. They succeeded in producing antibodies to denatured DNA by utilizing a complex consisting of denatured DNA and methylated bovine serum albumin (MBSA) (48).

RATIONALE

It is known that streptolysin S consists of two distinct portions, polypeptide and polyribonucleotide. The

polypeptide portion is an acidic polymer because it is rich in glutamic acid and has a low content of basic amino acids (41). In light of the work of Plescia et al. (48) with denatured DNA and methylated bovine serum albumin (MBSA) it seemed reasonable to try to make a complex of streptolysin S and MBSA, a basic protein, which presumably would interact electrostatically just like denatured DNA and MBSA. The MBSA would act as a carrier protein. In this way one might be able to make streptolysin S antigenic.

On addition of MBSA to streptolysin S a precipitate formed and experiments were then conducted to determine what happened to the streptolysin S hemolytic activity. Was the hemolytic activity precipitated? Did any hemolytic activity remain in the supernatant? If the hemolytic activity was precipitated, could it be recovered? If the last possibility were the case one could infer that the configuration of the streptolysin S molecule was maintained in an unaltered form in the precipitate.

An attempt was also made to produce antibodies to RNA and RNA core according to the method of Plescia et al. (48). Since RNA core is part of the polypeptide-polyribonucleotide complex which goes under the name of streptolysin S, it was hoped that such antibodies would cross react with streptolysin S and inhibit it. In this

regard it is noteworthy that streptolysin S is not commercially produced and its production is tedious and time-consuming. Most of the streptolysin S lots used in these studies were prepared in Dr. Taranta's laboratory. RNA and RNA core are both produced commercially, and therefore, are readily available.

Because of the success of Goodfriend et al. in production of antibodies to the low molecular weight polypeptides bradykinin and angiotensin with the use of soluble carbodiimides as coupling agents (23), and because streptolysin S is known to have a polypeptide portion, an attempt was made to produce antibodies to streptolysin S by immunizing a rabbit with streptolysin S coupled by 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride ("Ethyl CDI") to rabbit serum albumin (RSA). By analogy with Goodfriend's work it was assumed that the carbodiimide would act as a transient carrier for MBSA, followed by replacement of the carbodiimide by streptolysin S, and formation of a peptide bond between the latter and MBSA.

During the course of this investigation it was found that streptolysin S when passed through a Seitz filter was bound to the filter and could be eluted from it by solutions of high ionic strength. Because of this chance observation immunization attempts were made using a Seitz

filter impregnated with streptolysin S. The filter served as a "carrier" for the streptolysin S as well as an "adjuvant" (irritant).

METHODS AND MATERIALS

Streptolysin S: Streptolysin S was prepared by Worthington Biochemical Corp. and in Dr. Taranta's laboratory according to the method of Bernheimer (5) with modifications suggested by Ginsburg (19). The hemolysin was stored in the lyophilized state and was dissolved in streptolysin S buffer just before use.

Streptolysin S Buffer: Streptolysin S buffer was prepared according to the method described by Bernheimer (5).

Stock Erythrocyte Suspension: The stock erythrocyte suspension was prepared according to the method described by Bernheimer (3).

Methylated Bovine Serum Albumin: Methylated bovine serum albumin was prepared according to the method of Sueoka and Cheng (63).

Adjuvant: Complete Freund's Bacto Adjuvant was obtained from Difco Labs.

Measurement of Hemolytic Activity: The hemolytic activity was titrated according to the method of Bernheimer (3).

One unit of streptolysin S is defined as that amount of hemolysin which will cause 50% hemolysis of a .7% suspension of human red blood cells contained in 2 ml. of phosphate-buffered saline, pH 7, in 30 minutes at 37°C.

Standard Method for Titration of Serum Inhibitory Activity Against Streptolysin S: Serum inhibitory activity against streptolysin S was determined according to the method of Stollerman and Bernheimer (60).

Preparation of Ouchterlony Plates: (as in the laboratory of S. Halbert, and essentially as follows): 4.25 grams sodium chloride were dissolved in 500 ml. distilled water. 2.5 grams Ionagar N:2 were added and the suspension was boiled until all the agar was dissolved. Solid sodium veronal, 4.13 grams, was then added, followed by solid glycine, 7.5 grams. After the last two ingredients were dissolved the solution was cooled to 60°C. After 50 mgm. methiolate (1:10,000) was added, the pH was adjusted to 7.7 at 60°C with 5N HCl or 5N NaOH. The pH shifted to 7.4 on cooling to 20°C. The agar was stored in Erlenmeyer flasks and liquefied and poured into Petri dishes when needed. Holes were cut with a pattern cutter.

Modification of Farr Technique: The Farr technique has been published previously (12)(13). The essential requirement for use of the Farr technique is that the

antigen in question not be precipitated by saturated ammonium sulfate. Radioactive antigen which is customarily used in this technique has not been employed in the present study. Instead, the supposed antigen, streptolysin S, possesses a specific biologic activity of its own. Its presence can be detected by hemolytic titration.

Rabbits: All rabbits used in this study were New Zealand white male albinos, 7-8 pounds.

Ribonuclease: Ribonuclease was obtained from Worthington Biochemical Corp. (lot # R-547).

Trypsin: Trypsin was obtained from Worthington Biochemical Corp. (lot # TR558SF).

Rabbit Serum Albumin: Rabbit serum albumin was obtained from Pentex, Incorp. (lot # 9).

Carbodiimide: 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride ("Ethyl CDI") was obtained from the Ott Chemical Co.

Ribonucleic Acid: Ribonucleic acid (yeast) was obtained from Worthington Biochemical Corp. (lot # RNA 7DB).

Ribonucleic Acid Core: Ribonucleic acid core was obtained from Worthington Biochemical Corp. (lot # RNAC 632).

Millipore Filter: Millipore filters were obtained from the Millipore Filter Corp. Specifications were GSWP 047 00, GS 0.22u, white, plain, 47 mm.

Seitz Filter: Seitz filters were obtained from Republic Seitz Filter Corp. Specifications were S1 sterilizing #6 pore size 0.2u, diameter 60mm.

EXPERIMENTAL

Immunization with Streptolysin S and Methylated Bovine Serum Albumin: 10 mgm. MBSA was dissolved in .5 ml. distilled water in test tube #1 which was then placed in an ice bath. In test tube #2, 4 mgm. streptolysin S was dissolved in .5 ml. streptolysin S buffer which had been pre-cooled in an ice bath. The MBSA solution was added to the streptolysin S solution followed by the immediate formation of a milky white precipitate. This new suspension was titrated for hemolytic activity. The results of all titrations for injections in the SS-MBSA immunization experiment are in Table I.

One ml. of adjuvant was then added to the SS-MBSA suspension and a smooth emulsion was prepared by thorough mixing. The next step was the injection of a rabbit with this preparation. All injections were intramuscular between the scapulae. Although the original solutions totaled 2 ml. (.5 ml. streptolysin S in buffer plus .5

ml. MBSA in distilled water plus 1 ml. adjuvant) approximately 1.5 ml. remained after complete mixing with the adjuvant.

Table I

Rabbit	Injection Number	Units of Streptolysin S Before Addition of MBSA	Units of Streptolysin S After Addition of MBSA
1	1	76,000	less than 80
1	2	90,000	less than 80
1	3	96,000	less than 80
1	4	108,800	not done
3	1	42,000	290
3	2	105,000	550
3	3	125,000	750
3	4	108,800	not done
4	1	105,000	550
4	2	125,000	750
4	3	240,000	3,600
4	4	108,800	not done

In an alternate method for the preparation of streptolysin S for immunization the heat lability of the toxin was considered. This lability is not due to oxidation, and it is not due to surface inactivation. Bernheimer found that potassium ions protect streptolysin S against

thermal inactivation (6) thus decreasing its heat lability. Because of this observation KCl was utilized in some of the immunization attempts. The method was similar to that detailed above except for the following points. KCl was added to the streptolysin S solution before the MBSA solution was added. To the .5 ml. streptolysin S solution, 150 mgm. KCl was added. As can be seen in Table I, more hemolytic activity was preserved after the addition of MBSA when KCl was utilized (rabbits 3 and 4) than in the absence of KCl (rabbit 1). After this new solution was titrated for hemolytic activity the MBSA solution was added and the remainder of the procedure was completed (as detailed above).

Rabbit 1 was given a series of three injections of the SS-MBSA product. Rabbit 2 was used as a control and was injected with only MBSA. 10 mgm. MBSA was dissolved in 1 ml. distilled water. To this solution 1 ml. adjuvant was added and the two were mixed. Rabbits 3 and 4 were injected three times with streptolysin S to which KCl had been added plus MBSA. All injections were given at weekly intervals.

Each rabbit was bled before any injections had been given. From the date of the first injection the rabbits were bled daily (4 ml. each bleeding) for three weeks (until one week after the third injection had been given).

Daily bleedings were performed because we were not only interested in an antibody response but also in possible changes in streptolysin S inhibitor (SSI) levels. The serum from each sample was separated and was frozen at -4°C. After all sera were collected for a given rabbit, the anti-hemolytic activity of the pre- and post-injection sera was determined.

The results of the anti-hemolytic activity determinations are presented in Figure 1. It is clear from this data that there was no significant change in the anti-hemolytic activity of the post-injection sera when compared with the pre-injection serum.

With the above approach only those antibodies directed against the hemolytic site may be presumed to be detected. It is possible to hypothesize antibodies against other parts of the molecule which under the conditions of this system would go undetected since no precipitate is formed. Because of this possibility, other approaches were utilized in an effort to detect the presence of antibodies against streptolysin S.

The plate method of Ouchterlony gave the following results (Figure 2). The plate illustrated below is representative of all plates for post-injection sera from rabbits 1, 3, and 4.

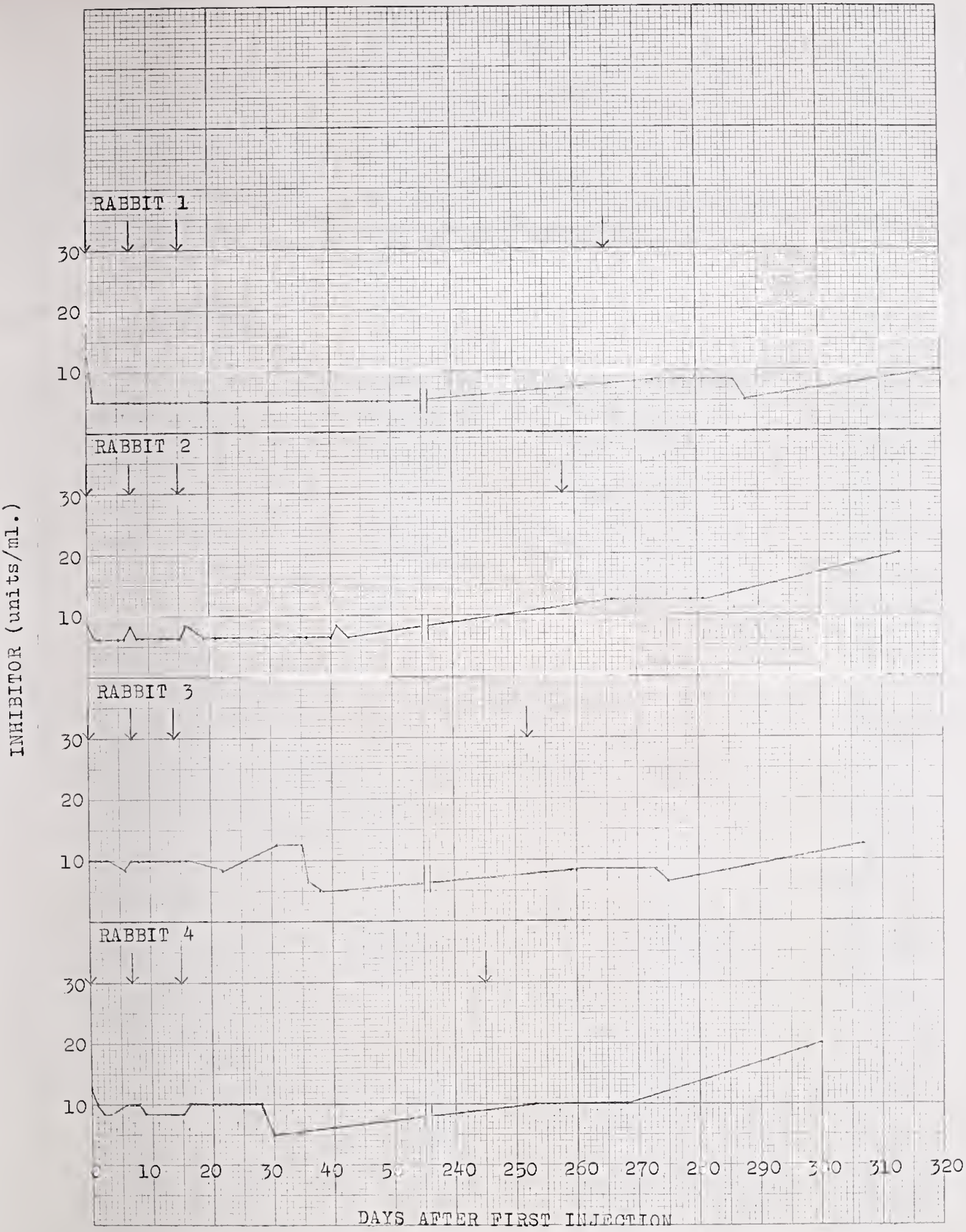

FIGURE 1 - Streptolysin S inhibitor levels in rabbits immunized with streptolysin S - MBSA complexes (1, 3 and 4) or with MBSA (2) in complete Freund's adjuvant.

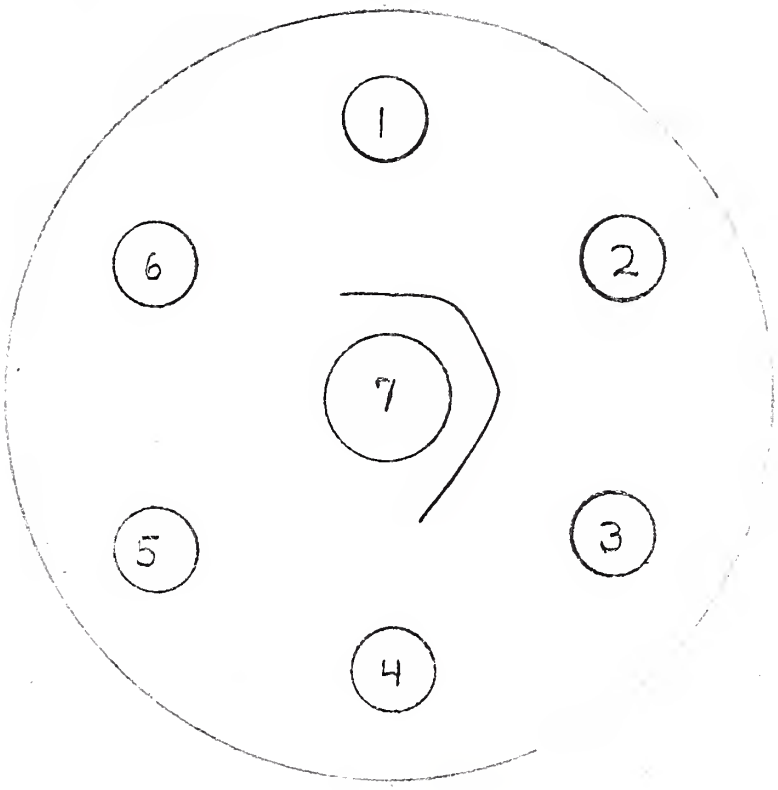

FIGURE 2--Ouchterlony plate showing the reactivity of serum (21 days after injection 4) from rabbit 4 injected with streptolysin S-MBSA complex: 1. streptolysin S, 10 mgm./ml.; 2. RNA core, 10 mgm./ml.; 3. RNA-se, 0.01 mgm./ml.; 4. RNA, 10 mgm./ml.; 5. MBSA, 10 mgm./ml.; 6. MBSA, 1 mgm./ml.; 7. Serum.

Precipitates formed between the center well (7) and wells 1, 2, and 3 with a reaction of identity between them. Since RNA-se is used in the preparation of RNA core and RNA core in the preparation of streptolysin S, the conclusion appears justified that the antibody here detected

is directed against RNA-se just as in the immunization experiments with RNA core to be described later. Using this system, no antibodies were detected in post-injection sera from rabbit 2. No precipitating antibodies directed against MBSA were present in any of the post-injection sera (rabbits 1 through 4).

To detect the possible presence of non-inhibiting and non-precipitating antibodies, a third method was used, a modification of the Farr technique (12)(13). With this method one may be able to precipitate such antibodies bound to streptolysin S. The protocol presented below was arrived at after several preliminary experiments. This experiment was performed with representative sera taken from rabbits 1, 3, and 4. Tubes 1 through 6' were prepared as indicated in Table II.

After thorough mixing, tubes 1 through 6 were incubated at 4°C for 24 hours while tubes 1' through 6' were incubated at 37°C for 30 minutes. After incubation both sets of tubes were treated similarly. 2 ml. saturated ammonium sulfate solution was added to tubes 1, 3, and 5 and 1', 3', and 5'. At the same time 2 ml. streptolysin S buffer was added to tubes 2, 4, and 6 and 2', 4', and 6'. All of these tubes were mixed, incubated at 4°C for 30 minutes and then centrifuged at 1500 rpm for ten minutes. The supernatants were then titrated for hemolytic

activity. The results of a typical experiment are presented in Table III (on page 34).

Table II

Tube	Streptolysin S 600 units/ml.	Normal Serum*	Post-injection Serum*	Streptolysin S Buffer
1	1 ml.	1 ml.	---	---
1'	1 ml.	1 ml.	---	---
2	1 ml.	1 ml.	---	---
2'	1 ml.	1 ml.	---	---
3	1 ml.	---	---	1 ml.
3'	1 ml.	---	---	1 ml.
4	1 ml.	---	---	1 ml.
4'	1 ml.	---	---	1 ml.
5	1 ml.	---	1 ml.	---
5'	1 ml.	---	1 ml.	---
6	1 ml.	---	1 ml.	---
6'	1 ml.	---	1 ml.	---

* Inactivated

Table III

Part A--Incubated at 4°C for 24 hours.

Tube	Contents	Original Units of Streptolysin S	Final Units of Streptolysin S	Percent Recovery
1	Normal serum	600	210	35%
2	Normal serum	600	320	53%
3	Streptolysin S buffer	600	385	64%
4	Streptolysin S buffer	600	490	82%
5	Post-injection serum*	600	320	53%
6	Post-injection serum	600	350	58%

Part B--Incubated at 37°C for 30 minutes.

Tube	Contents	Original Units of Streptolysin S	Final Units of Streptolysin S	Percent Recovery
1'	Normal serum	600	240	40%
2'	Normal serum	600	280	47%
3'	Streptolysin S buffer	600	190	32%
4'	Streptolysin S buffer	600	255	42%
5'	Post-injection serum	600	230	38%
6'	Post-injection serum	600	330	55%

* Rabbit 4, 21 days after injection 4.

The basic point to be noted is that there was no decrease in recovery of hemolytic activity in the immune serum with respect to recovery in the normal serum. Although normal serum and immune serum with saturated ammonium sulfate solution yielded a lower recovery of hemolytic activity than did comparable tubes without ammonium sulfate, the same phenomenon was observed in the control tubes which contained streptolysin S buffer in place of serum (tubes 3 and 3'). Therefore, this decrease in hemolytic activity cannot be explained on the basis of precipitation of complexes consisting of serum constituents (antibody or otherwise) and streptolysin S by ammonium sulfate solution.

Precipitation of Streptolysin S by Methylated Bovine Serum Albumin and Its Recovery from the Precipitate: Because of the absence of an antibody response in the previous experiment, the question arose whether streptolysin S was destroyed in the preparation of the SS-MBSA complex. This possibility was explored by the following experiment.

10 mgm. MBSA was dissolved in 0.5 ml. distilled water and 4 mgm. streptolysin S (19,600 units/mgm., 78,400

units total) was dissolved in 0.5 ml. streptolysin S buffer. The solutions were prepared in an ice bath and on mixing the two a precipitate formed immediately. The suspension was centrifuged and the precipitate washed twice with 4 ml. streptolysin S buffer (diluted 1:2 with distilled water). The hemolytic activity of the supernatant and the washings was less than 20 units.

The precipitate was resuspended in 1.5 ml. streptolysin S buffer and divided into three equal parts (Table IV).

Table IV

Tube	Resuspended Ppt.	Additional Contents
1	.5 ml.	2 ml. streptolysin S buffer
2	.5 ml.	2 ml. rabbit serum (unheated)
3	.5 ml.	2 ml. phosphate buffer, pH 9.5

Controls

Tube	Streptolysin S Buffer	Additional Contents
4	.5 ml.	2 ml. streptolysin S buffer
5	.5 ml.	2 ml. rabbit serum (unheated)
6	.5 ml.	2 ml. phosphate buffer, pH 9.5

All suspensions were incubated for one hour at 37°C and were then centrifuged. Hemolytic activity of the supernatants was assayed with red blood cells from the rabbit which had provided the serum (to avoid immune

hemolysis).

The hemolytic activity of tubes 1 through 6 is presented in Table V.

Table V

Tube	Hemolytic Activity (units/ml.)
1	less than 20*
2	137
3	6,850**
4	0
5	0
6	0

* No hemolysis at 1:20 dilution.

** 6,850 (hemolytic units/ml.) x 2.5 (ml./test tube) x 3 (number of test tubes) = 51,375 units. Thus, 65% of the initial activity can be recovered with the extraction procedure used for tube 3.

The finding that streptolysin S hemolytic activity could be recovered in such high yield from the precipitate which formed when streptolysin S and MBSA were mixed is direct evidence that the bulk of streptolysin S was not destroyed in the preparation of the SS-MBSA complex.

Determination of Optimal Ratio of Ribonucleic Acid, Ribonucleic Acid Core, and Streptolysin S to Methylated Bovine Serum Albumin for Immunization Procedures: The following experiments were performed to establish whether the ratio of streptolysin S to MBSA utilized in the SS-MBSA immunization experiment was an ideal one, i.e., whether the complex prepared was as rich as possible in streptolysin S. Since streptolysin S was available in very limited amounts immunization efforts with complexes consisting of MBSA with RNA and RNA core respectively were also planned. Therefore, additional experiments were performed to determine optimal ratios of RNA and RNA core to MBSA for immunization.

Varying amounts of RNA dissolved in .5 ml. streptolysin S buffer were added to each of seven test tubes as indicated in Table VI. On the addition of a constant amount of MBSA to each tube a precipitate formed immediately. The new suspension was incubated in an ice bath for ten minutes. Then the tubes were centrifuged, and the optical density of the supernatants at 260 mμ and 280 mμ was determined (Table VI). The object of this procedure was to find the minimum amount of RNA necessary to combine with all of the MBSA (said another way, to find the largest amount of RNA which could be completely precipitated by .5 mgm. MBSA). By determining the O.D. of the supernatants at 260 mμ and 280 mμ any MBSA or RNA remaining in the supernatants after

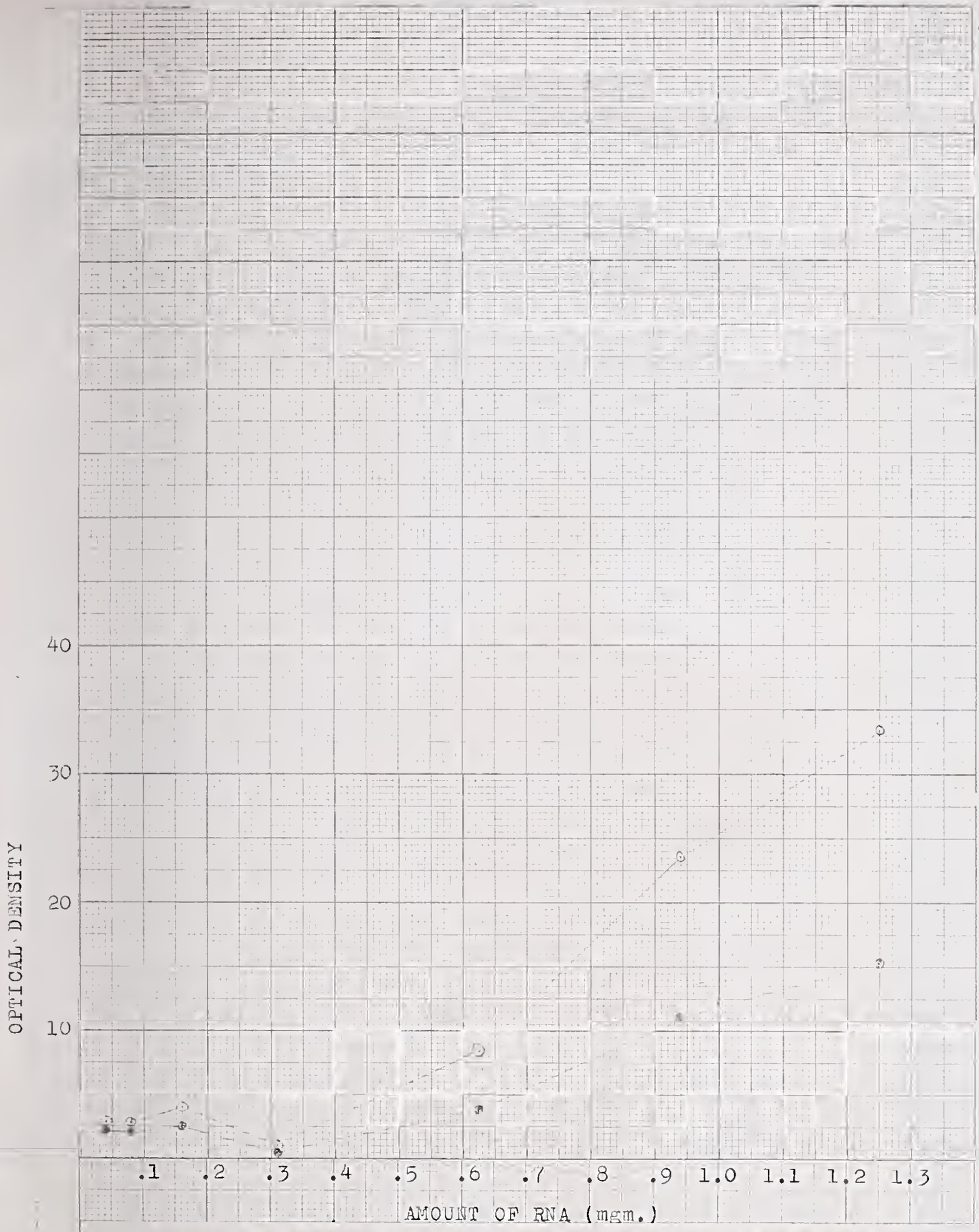

FIGURE 3 - Optical density at 260 mμ and 280 mμ of super natants of RNA - MBSA precipitates. The amount of MBSA was kept constant (.5 mgm.). The amount of RNA varied and is indicated on the abscissa: optical density at 260 mμ, ○—○; optical density at 280 mμ, ⊙—⊙.

hemolytic activity (Table VIII).

Table VII

Tube	Mgm. RNA core in .2 ml. SS buffer	Mgm. MBSA in .2 ml. distilled water	O.D. at 260 mμ	O.D. at 280 mμ
A	1.25	.5	52.4	24.0
B	.94	.5	36.4	16.6
C	.625	.5	21.6	9.8
D	.31	.5	7.3	3.2
E	.16	.5	3.4	1.8
F	.08	.5	2.5	1.7
G	.04	.5	3.3	2.1

Table VIII

Tube	Units of SS in .2 ml. SS buffer	Mgm. SS	Mgm. MBSA in .2 ml. distilled water	Recovery of SS in units	O.D. at 260 mμ	O.D. at 280 mμ
A	20,000	1.25	.5	8,000	29.2	14.8
B	15,000	.94	.5	4,500	21.6	11.0
C	10,000	.625	.5	180	10.5	5.2
D	5,000	.31	.5	8	3.4	2.0
E	2,500	.16	.5	9.2	2.3	1.5
F	1,250	.08	.5	33.2	2.2	1.7
G	625	.04	.5	36	2.4	2.0

The object was to determine the minimum amount of streptolysin

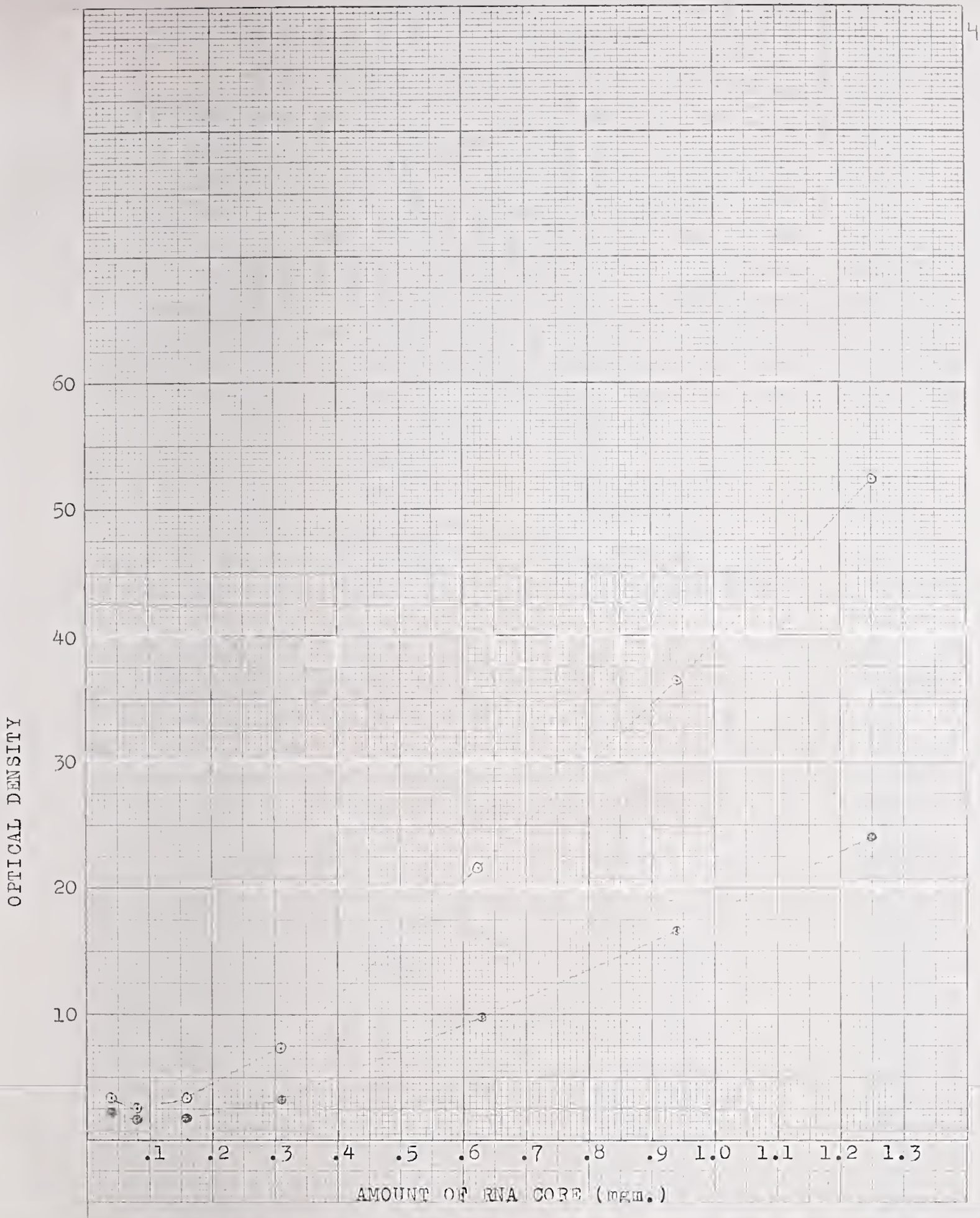

FIGURE 4 - Optical density at 260 mμ and 280 mμ of supernatants of RNA core - MBSA precipitates. The amount of MBSA was kept constant (.5 mgm.). The amount of RNA core varied and is indicated on the abscissa: optical density at 260 mμ, o—o ; optical density at 280 mμ, ⊙ ⊙.

S necessary to combine with all of the MBSA (said another way, to find the largest amount of streptolysin S which could be precipitated by .5 mgm. MBSA). As seen in Figure 5, the low point with respect to hemolytic activity present in the supernatants falls at .31 mgm. streptolysin S (tube D). On the basis of this experiment the optimal ratio of streptolysin S to MBSA was calculated to be .3 mgm. streptolysin S to .5 mgm. MBSA or .6 : 1.0.

The optimal ratio of streptolysin S to MBSA (also RNA and RNA core to MBSA) arrived at in this experiment (.6 to 1.0) is similar enough to the ratio employed in the SS-MBSA immunization experiment (.4 to 1.0) to conclude that the ratio used was an adequate one. On the basis of these results the immunization efforts with RNA and RNA core will utilize the ratio .6 to 1.0 (RNA:MBSA and RNA core:MBSA).

Immunization with Ribonucleic Acid: 80 mgm. MBSA was dissolved in .75 ml. distilled water and 50 mgm. RNA was dissolved in .75 ml. streptolysin S buffer, both at room temperature. After the MBSA solution was added to the RNA solution a milky white precipitate formed immediately. The new product (1.5 ml.) was mixed with 1.5 ml. complete Freund's adjuvant. The emulsion was injected

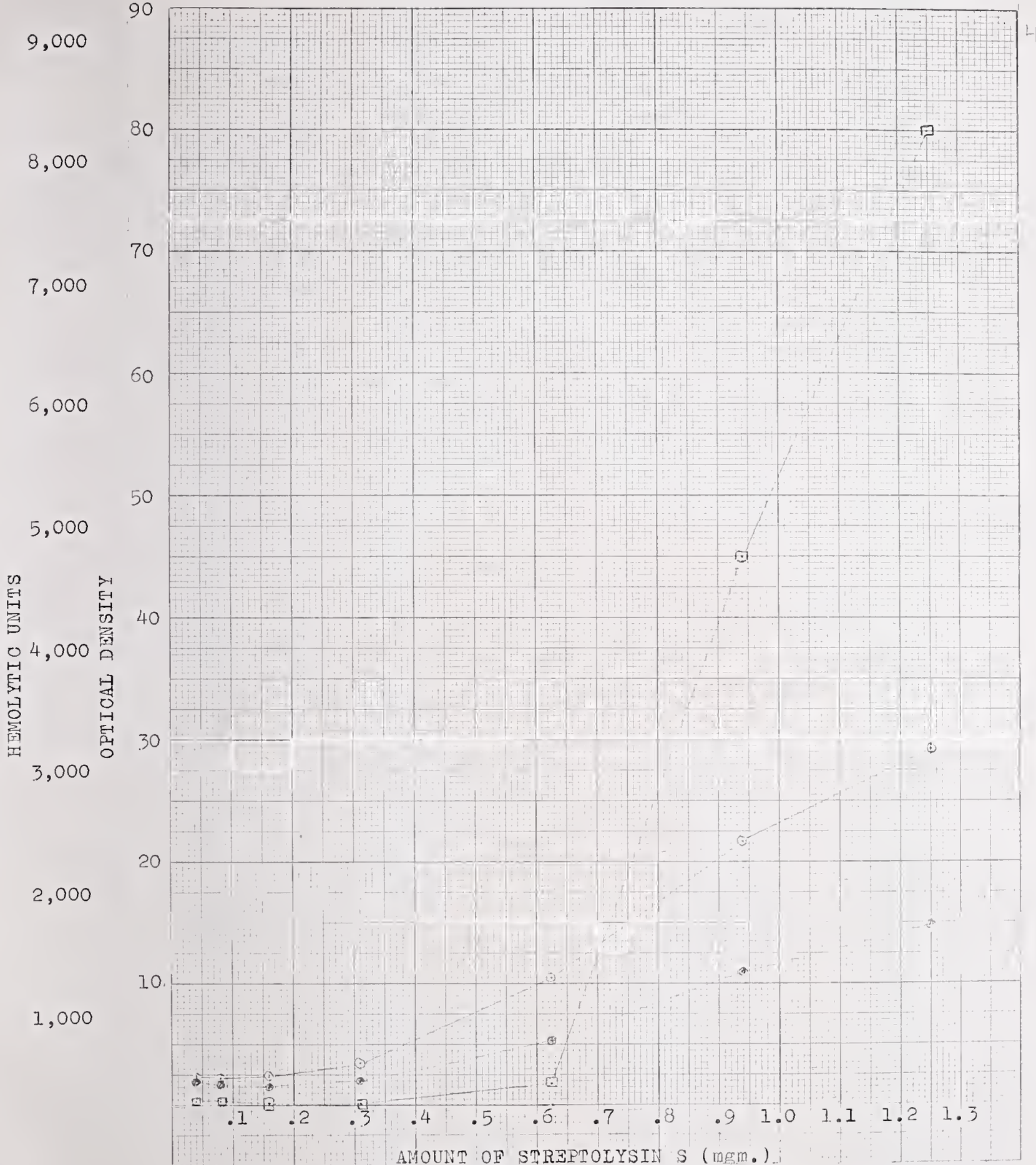

FIGURE 5 - Optical density at 260 mμ and 280 mμ and hemolytic activity of supernatants of streptolysin S - MBSA precipitates. The amount of MBSA was kept constant (.5 mgm.). The amount of streptolysin S varied and is indicated on the abscissa: optical density at 260 mμ, ○—○ ; optical density at 280 mμ, ●—●; hemolytic units, □—□.

into each of two rabbits (333, 334) as follows: (all injections were subcutaneous) .5 ml. in back and front of neck; .5 ml. in each of four foot pads. Both rabbits were bled pre- and post-injection (day 0, 8, 16, 26, 34, 37, 48).

All sera were tested for their titer of inhibitory activity against streptolysin S by the standard method. The results presented in Figure 6 show only minor variations in inhibitory titer which are not suggestive of an antibody response.

All sera were tested on Ouchterlony plates as illustrated in Figure 7. Under these conditions the post-injection sera from rabbit 333 showed no antibody response. Pre-injection serum from rabbit 334 showed a line of precipitation with RNA core. On the eighth post-injection day the line of precipitation between serum and RNA core took longer to appear. On the ninth post-injection day a line appeared between the serum and the RNA which by day 16 had become more evident than the RNA core line. Finally by day 34 a suggestion of a double precipitating system appeared against RNA and RNA core (with a reaction of identity in each of the lines). This would indicate the presence of antibodies against two distinct constituents of both RNA and RNA core.

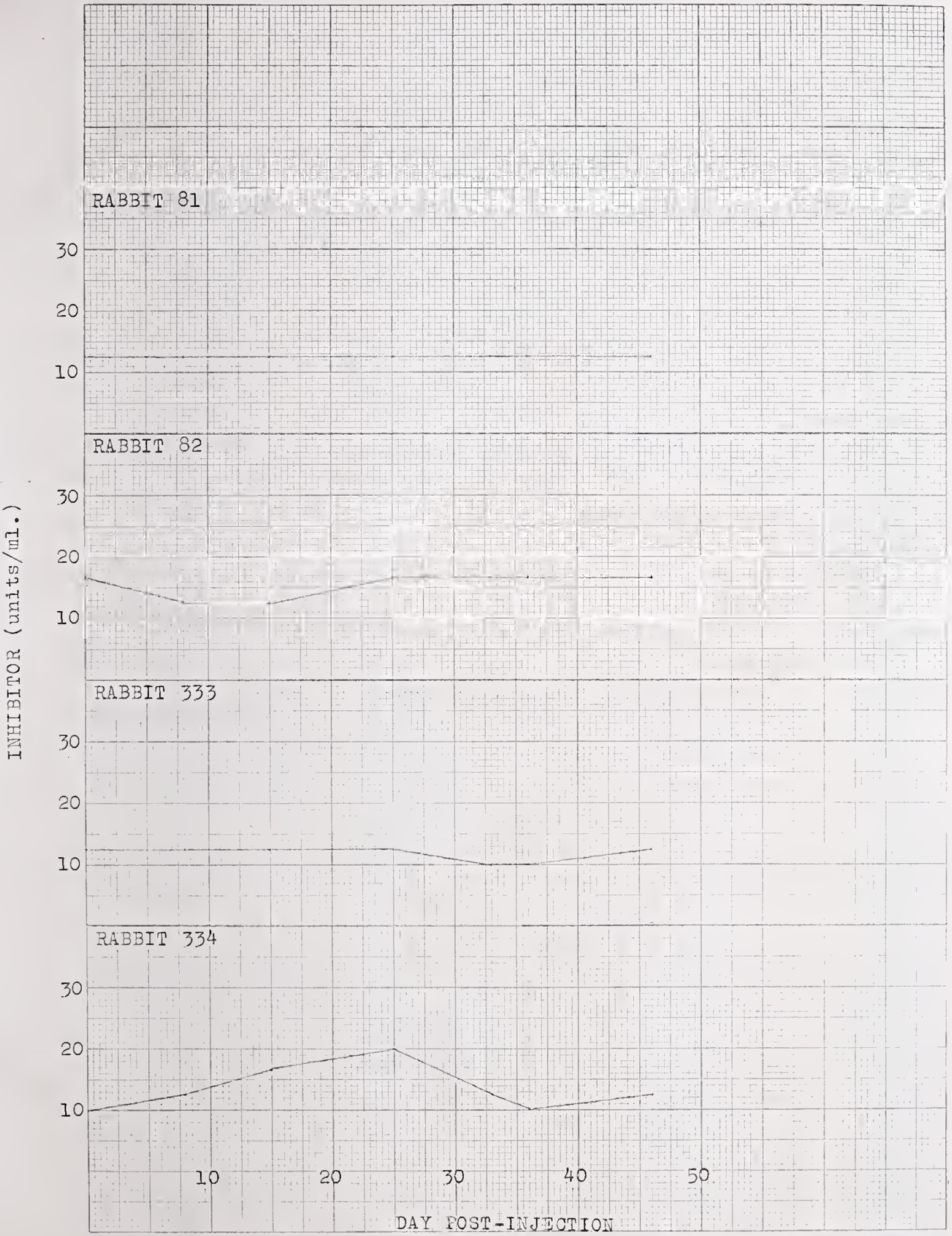

FIGURE 6 - Streptolysin S inhibitor levels in rabbits immunized with RNA core - MBSA complexes (81 and 82) or with RNA - MBSA complexes (333 and 334) in complete Freund's adjuvant.

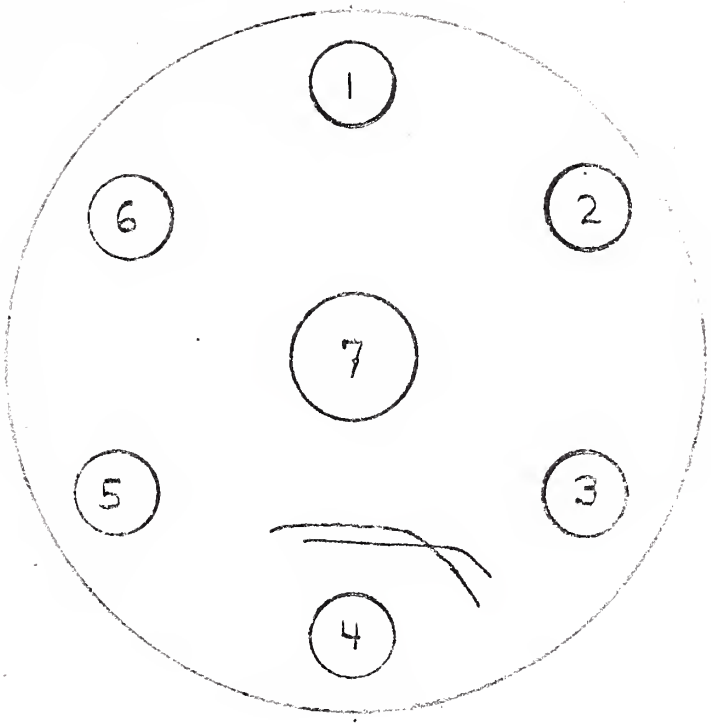

FIGURE 7--Ouchterlony plate showing the reactivity of serum (post-injection day 34) from rabbits 333 and 334, both injected with RNA-MBSA complex: 1. serum, rabbit 333; 2. RNA core, 10 mgm./ml.; 3. RNA core, 10 mgm./ml.; 4. serum, rabbit 334; 5. RNA-se .01 mgm./ml.; 6. RNA-se, .01 mgm./ml.; 7. RNA, 10 mgm./ml.

The first two rabbits immunized with RNA died a few months post-injection. Four more rabbits were immunized exactly as described above. The sera were tested for their titer of inhibitory activity against streptolysin S by the standard method. The results are not suggestive of an antibody response. Ouchterlony plates showed no evidence of an antibody response against RNA.

Immunization with Ribonucleic Acid Core: The details of this procedure were the same as those in the immunization with ribonucleic acid except for the substitution of RNA core for RNA (rabbits 81 and 82).

All sera were tested for their inhibitory titer against streptolysin S by the standard method. The results presented in Figure 6 show only minor variations in the inhibitory titer which are not suggestive of an antibody response.

All sera were tested on Ouchterlony plates as illustrated in Figure 8. Under these conditions both rabbits showed a line of precipitation between serum and RNA core and serum and RNA-se which was present on the twenty-sixth post-injection day in rabbit 81 and on the sixteenth post-injection day in rabbit 82. These lines for both rabbits showed a reaction of identity. Thus, the antibody formed was against a constituent present in both RNA core and RNA-se. The most likely possibility is that the antibody was against RNA-se which is used in the preparation of RNA core and, therefore, is a likely contaminant.

The two rabbits immunized with RNA core (81 and 82) were re-injected with an RNA core - MBSA complex six months after the initial set of injections and an additional two rabbits were given their first set of injections. The rabbits were bled at three and four weeks post-injection

and all sera were tested for their inhibitory titer against streptolysin S by the standard method. The results are not suggestive of an antibody response.

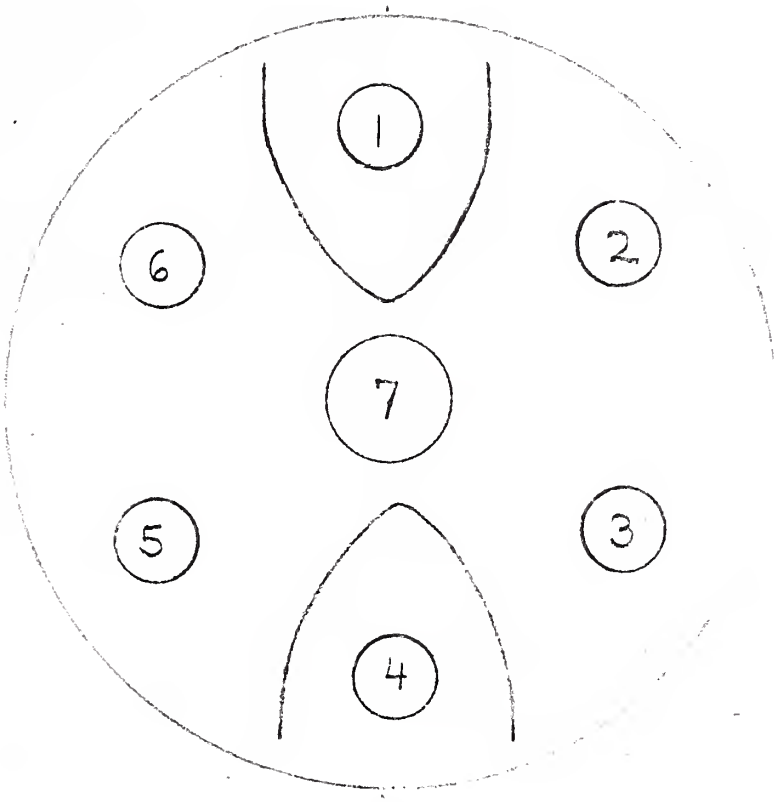

FIGURE 8--Ouchterlony plate showing the reactivity of serum (post-injection day 26) from rabbit 81 and of serum (post-injection day 16) from rabbit 82, both injected with RNA core - MBSA complex: 1. serum, rabbit 81; 2. RNA core, 10 mgm./ml.; 3. RNA core, 10 mgm./ml.; 4. serum, rabbit 82; 5. RNA-se, 0.1 mgm./ml.; 6. RNA-se, 0.1 mgm./ml.; 7. RNA, 10 mgm./ml.

All sera were tested on Ouchterlony plates and showed lines of precipitation with RNA core and RNA-se (Figure 9). These lines gave a reaction of identity. As stated previously the most likely possibility is that the antibody was against RNA-se.

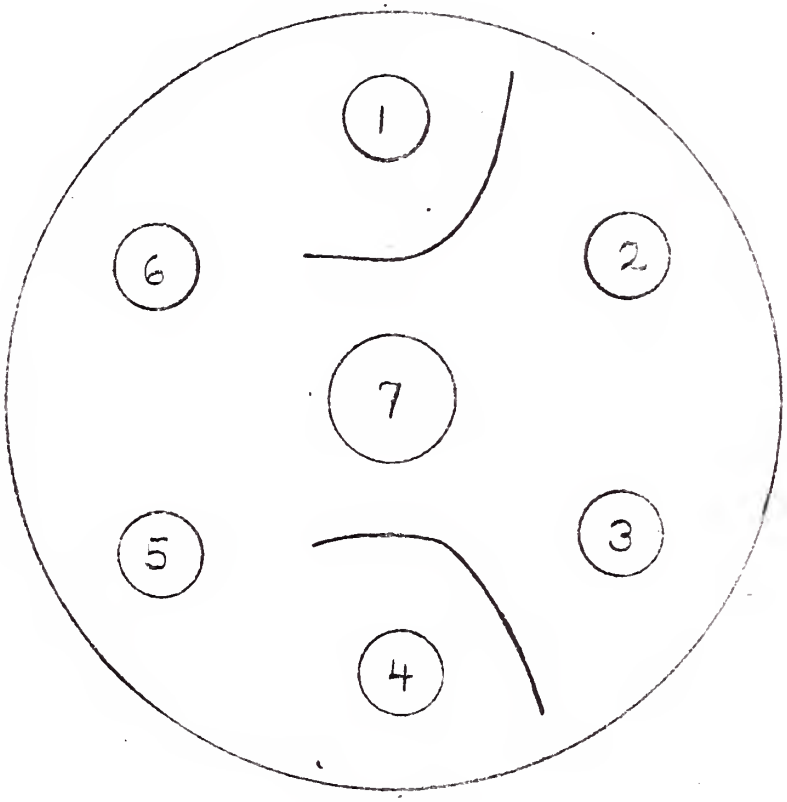

FIGURE 9--Ouchterlony plate showing the reactivity of serum (three and four weeks post-injection) from rabbit 82, injected with RNA core - MBSA complex: 1. post-injection serum, day 21; 2. RNA-se, 0.1 mgm./ml.; 3. RNA-se, 0.1 mgm./ml.; 4. post-injection serun, day 28; 5. RNA, 10 mgm./ml.; 6. RNA, 10 mgm./ml.; 7. RNA core, 10 mgm./ml.

Immunization with Streptolysin S and Rabbit Serum Albumin Coupled by a Carbodiimide: 10 mgm. rabbit serum albumin (RSA) and 10 mgm. streptolysin S were dissolved in 0.5 ml. distilled water in an ice bath. To this solution .25 ml. water containing 200 mgm. ethyl CDI was added. The reaction between ethyl CDI and the streptolysin S - RSA mixture was allowed to take place at room temperature with gentle agitation for 30 minutes. The reaction was terminated by dialysis against distilled water for 24 hours in the cold. Indirect evidence of successful conjugation was the formation of a precipitate within 24 hours. Both the precipitate and the soluble material were suspended in an equal amount of Freund's adjuvant and the resulting emulsion was used for immunization.

A single rabbit was injected intramuscularly and subcutaneously twice, 31 days apart. The rabbit was bled before the first injection and on post-injection days 21, 31, and 50. In an effort to demonstrate antibodies directed against the hemolytic site of streptolysin S, the anti-hemolytic activity of the pre- and post-injection sera was determined. There was no detectable increase in the anti-hemolytic activity of the post-injection sera when compared with the pre-injection serum.

A second attempt to detect antibodies to streptolysin S

utilized gel diffusion on Ouchterlony plates. With this method one could identify any precipitating antibody to streptolysin S whether or not the antibody was directed against the hemolytic portion of the molecule. The results of the gel diffusion test are presented in Figures 10 and 11.

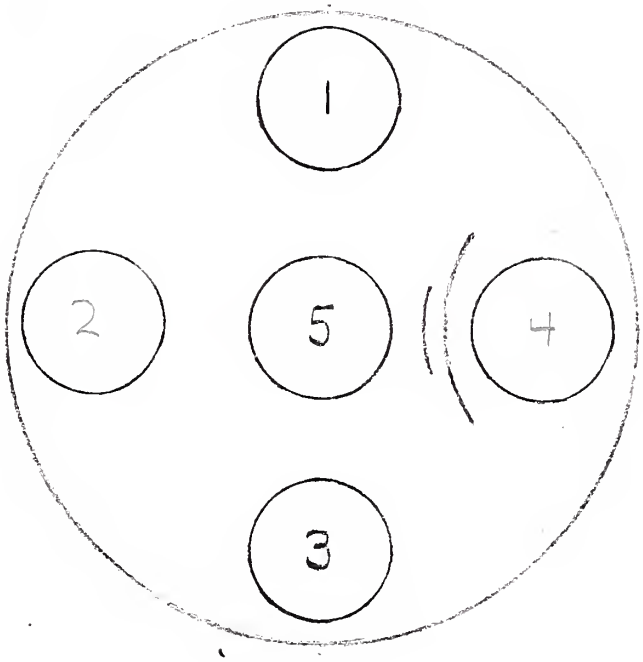

FIGURE 10--Ouchterlony plate (I) showing the reactivity of serum from a rabbit injected with streptolysin S - RSA complex: 1. pre-injection serum; 2. post-injection serum, day 21; 3. post-injection serum, day 31; 4. post-injection serum, day 50; 5. streptolysin S, 5 mgm./ml., Worthington Biochemical Corp.

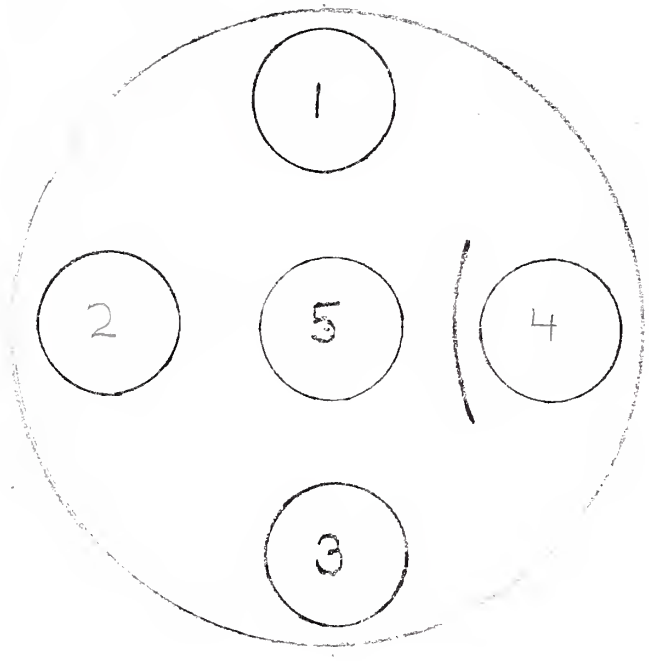

FIGURE 11--Ouchterlony plate (II) showing the reactivity of serum from a rabbit injected with streptolysin S - RSA complex: 1. pre-injection serum; 2. post-injection serum, day 21; 3. post-injection serum, day 31; 4. post-injection serum, day 50; 5. streptolysin S, 5 mgm./ml., Dr. Taranta's laboratory.

There were two bands of precipitation on Plate I between the central well (5) and well 4. Plate II had one band of precipitation between the central well (5) and well 4. There were no other lines of precipitation on either plate. Thus, antibodies were present in serum from post-injection day 50 against two distinct antigens present in

the Worthington Biochemical Corp. streptolysin S preparation. This same serum contained antibodies against one antigen present in the streptolysin S prepared in Dr. Taranta's laboratory. To find out whether one of these antigens was streptolysin S itself, the following experiment was carried out.

Streptolysin S was added to pre- and post-injection sera in an amount sufficient to form a visible precipitate in the latter. After incubation in the cold overnight, the precipitates were centrifuged out and the supernatants were titrated for hemolytic activity. There was no decrease of hemolytic activity in the supernatant of the post-injection serum as compared to that of the pre-injection serum. Therefore, it was concluded that the antibodies present in the post-injection serum were not against streptolysin S itself, but rather against impurities present in the streptolysin S preparations used. (By analogy with the results obtained on immunization with the streptolysin S-MBSA complex and with the RNA core-MBSA complex, one may surmise that one of the antibodies detected in the present experiment was directed against ribonuclease.)

Seitz Filtration of Streptolysin S and Extraction of Streptolysin S Hemolytic Activity by High Ionic Strength Solutions: 35 ml. non-dialyzed streptolysin S was passed through a Seitz filter in 5 ml. aliquots. This was followed by 30 5 ml. washings with the medium used for the preparation of streptolysin S less maltose, cysteine and RNA core. Finally eight 5 ml. aliquots of each of the following solutions were passed through the filter successively: medium described above plus .5% NaCl, 1% NaCl, 2% NaCl, 4% NaCl, 8% NaCl, 16% NaCl and a saturated solution of NaCl respectively. Each 5 ml. aliquot was collected separately and titrated for hemolytic activity. Then the optical density was determined at 260 mμ and 280 mμ. The results of a typical experiment are presented in Figure 12.

The total amount of hemolytic activity passed through the Seitz filter was 224,000 units. The recovery in the eluates was 126,500 units or 56%. The recovery of 260 mμ absorbing material was 93% and the recovery of 280 mμ absorbing material was 54%.

Immunization with Streptolysin S Bound to a Seitz Filter: A Seitz filter with streptolysin S bound to it was implanted intraperitoneally in a series of eight

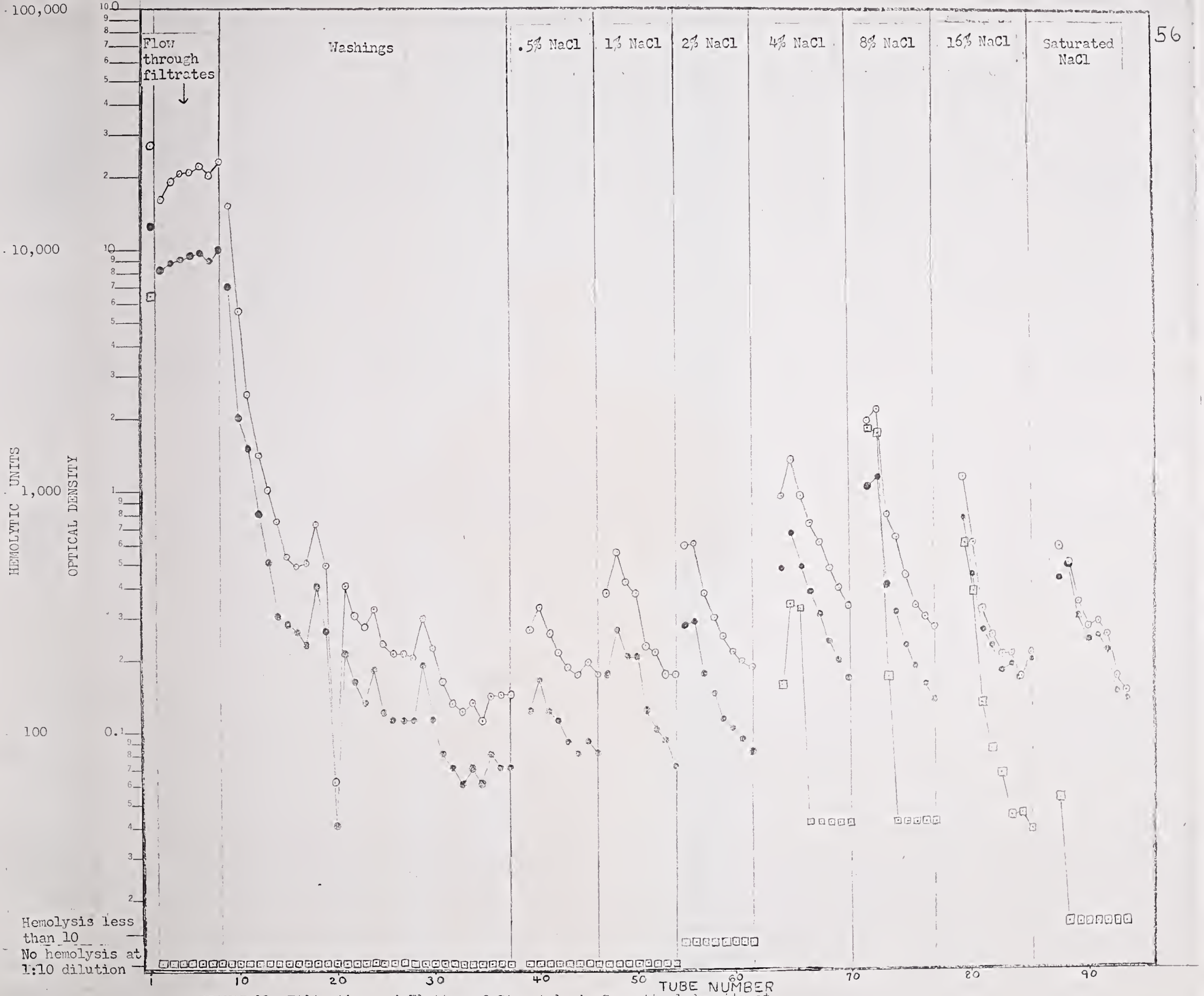

FIGURE 12- Filtration and Elution of Streptolysin S: optical density at 260 mμ, o——o ; optical density at 280 mμ, ●——● ; hemolytic units, □——□. Tube 1 is unfiltered streptolysin S.

rabbits. A control rabbit was implanted with only a filter free of streptolysin S. The details of the procedure are presented below. See Table IX for the number of units of streptolysin S bound to each filter.

Table IX

Rabbit	Units of Streptolysin S Implanted
327	292,000
328	190,000
329	142,000
330	147,000
331*	146,000 (intraperitoneal)
	63,000 (subcutaneous)
332*	146,000
335	132,000
342	Control
343	145,600

The following procedure was conducted under sterile conditions (except for rabbits 331 and 332). For each rabbit an average of 30 ml. non-dialyzed streptolysin S was passed through a sterile Millipore filter to rid the streptolysin S preparation of any streptococci still pre-

* Preparation of the Seitz filter for these rabbits was not performed under sterile conditions.

sent. The streptolysin S was then passed through a sterile Seitz filter in 5 ml. aliquots. All solutions used for washing were first passed through a sterile Millipore filter. After the streptolysin S, 30 5 ml. washings with the medium described in the previous section were passed through the Seitz filter followed by eight 5 ml. aliquots of the same medium with .5% Nacl added and eight with 1% NaCl added. All solutions passed through the filter were kept in an ice bath before use. All filtrates were disposed of.

Under sterile conditions the filter was mashed with a mortar and pestle in 20 ml. chilled streptolysin S buffer and implanted intraperitoneally in a rabbit. All rabbits were bled pre- and post-implantation and were given prophylactic antibiotics post-operatively.

The results of these attempts at immunization are presented in Figures 13 and 14. After implantation streptolysin S inhibitor levels rose in rabbits 330, 331 and 332 (Table X). There was a transient opalescence in the post-implantation sera from rabbits 328, 329, 330, 331 and 332 (Figure 13).

If one compares SSI levels and opalescence data for individual sera as presented in Figure 13, it is evident that (1) opalescence occurred in sera from rabbits 328 and 329 without a concomitant rise in SSI, (2) opalescence

occurred in sera from rabbits 331 and 332 with a concomitant rise in SSI and (3) a rise in SSI occurred in serum from rabbit 330 without the simultaneous occurrence of opalescence.

Table X

Rabbit	Streptolysin S Inhibitor (units/ml.) Pre-implantation	Peak Value Post-implantation	Percent Increase
330	12.5	33	165%
331	10	666	6500%
332	10	33	230%

On Ouchterlony plates all anti-sera showed no reaction with RNA core or RNA-se. Most early post-implantation sera reacted with non-dialyzed streptolysin S (Figure 15). One pre-implantation serum (rabbit 343) and several post-implantation sera from the control rabbit also reacted with the non-dialyzed streptolysin S. Since this precipitation reaction occurred so soon after the implantation it could not be due to an antibody but could perhaps be due to an acute phase reactant.

There were three rabbits who died on whom a bleeding was performed on the day preceding death (rabbits 328, 329 and 332). The sera from two of these three bleedings clotted after the initial clot had been removed (rabbits

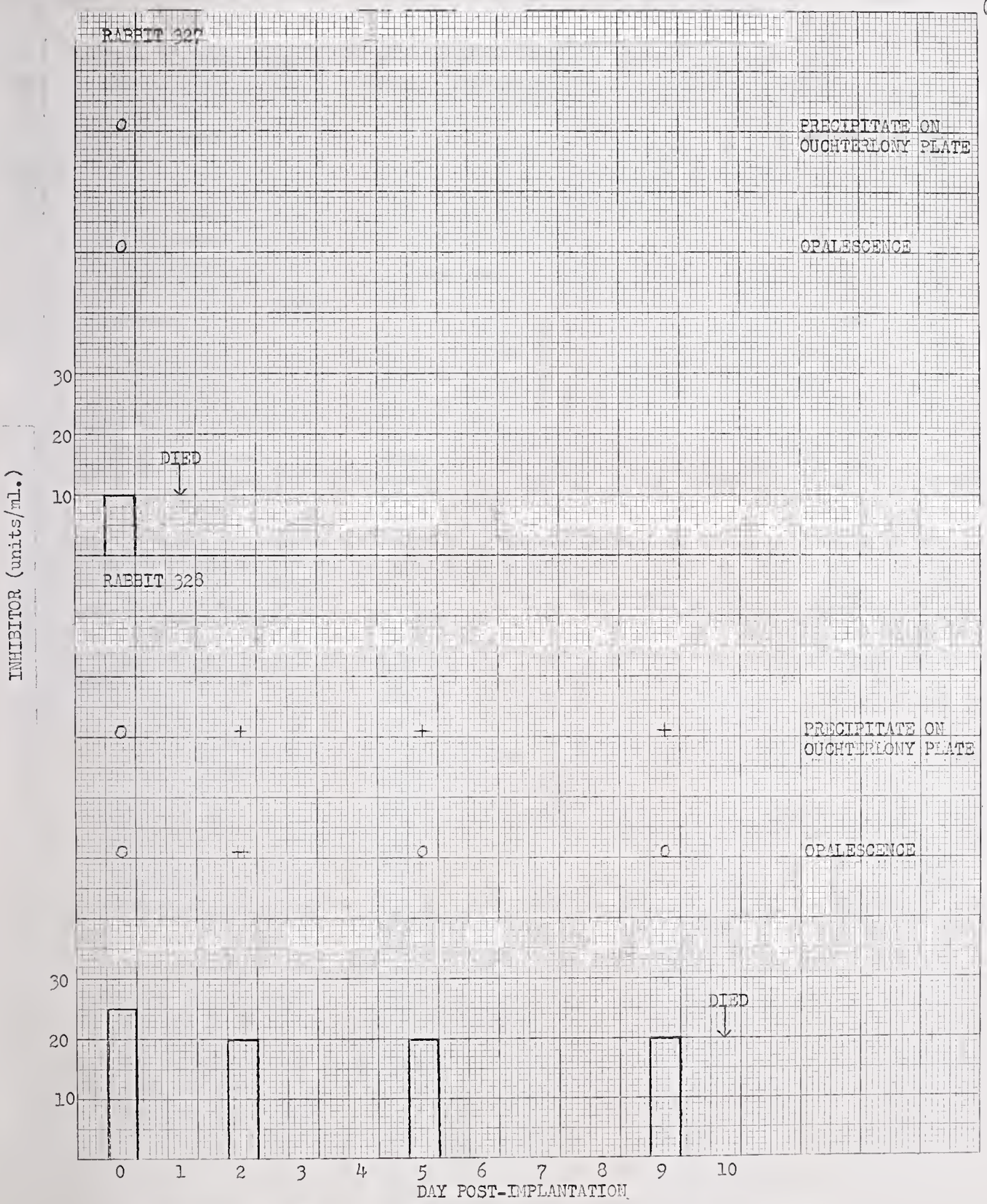

FIGURE 13- Serum inhibitor is shown in bar form; opalescence, 0 - 4+, and precipitation on Ouchterlony plate of serum against streptolysin S, 0/+, are shown above.

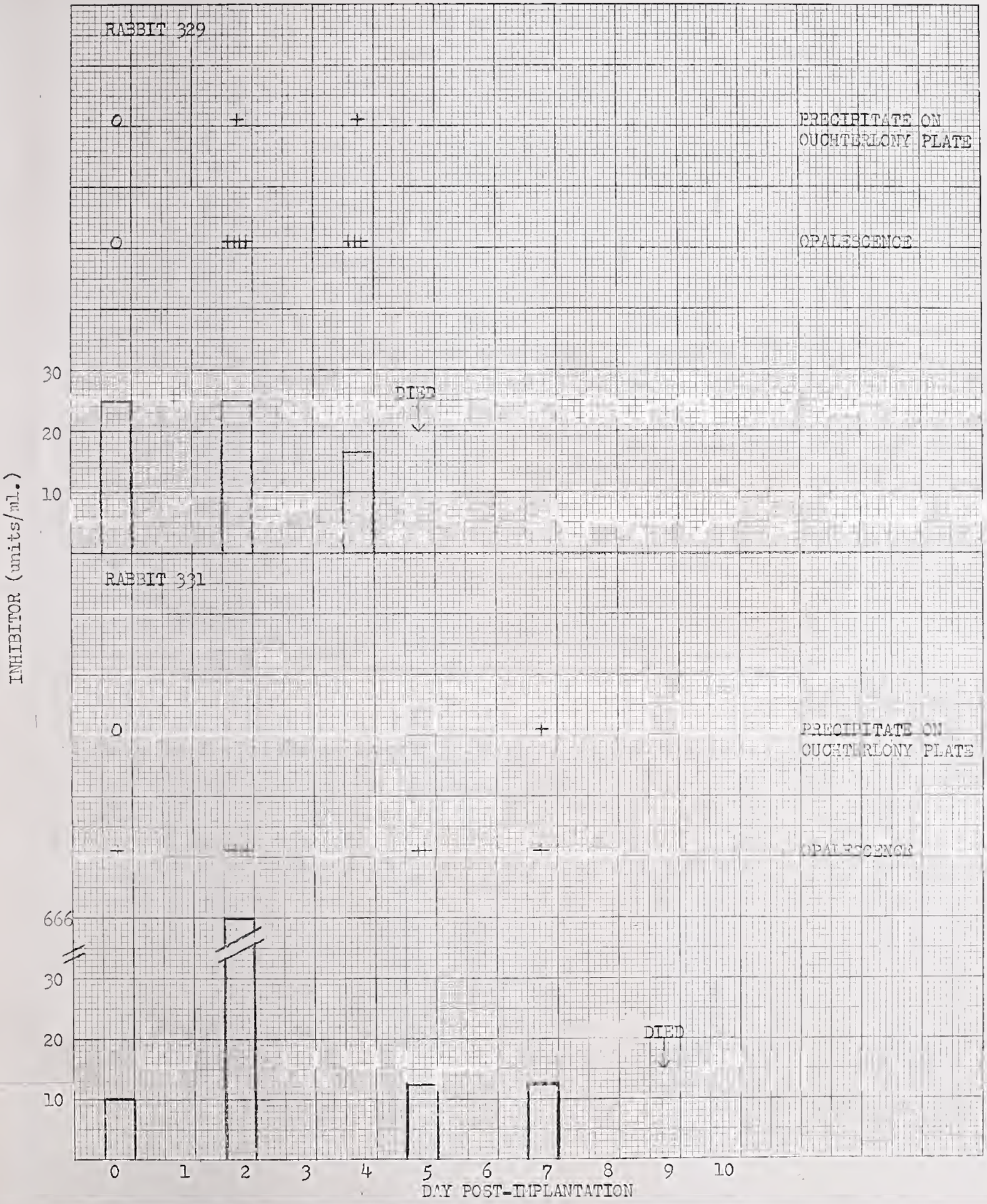

FIGURE 13- Continued.

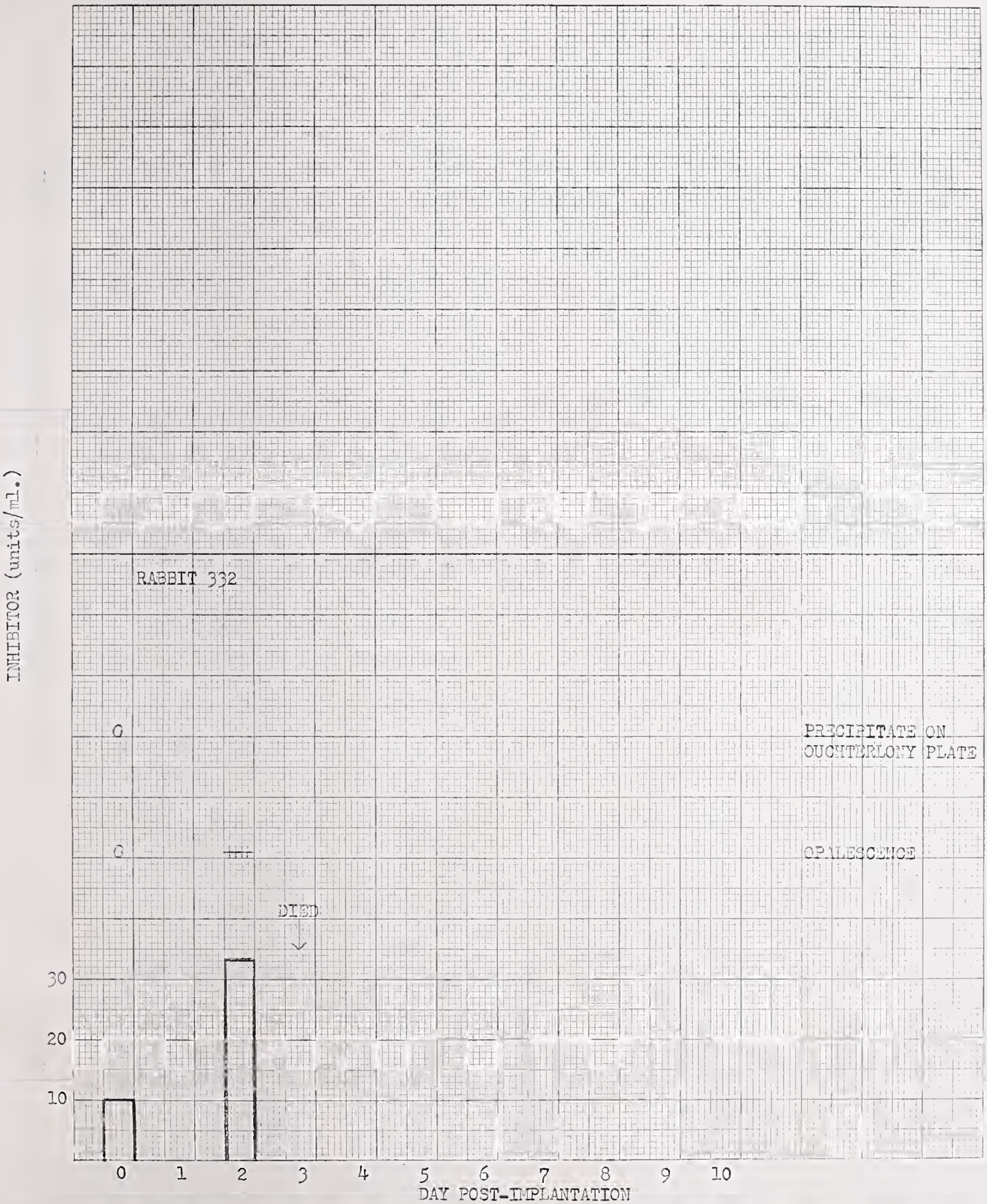

FIGURE 13- Continued.

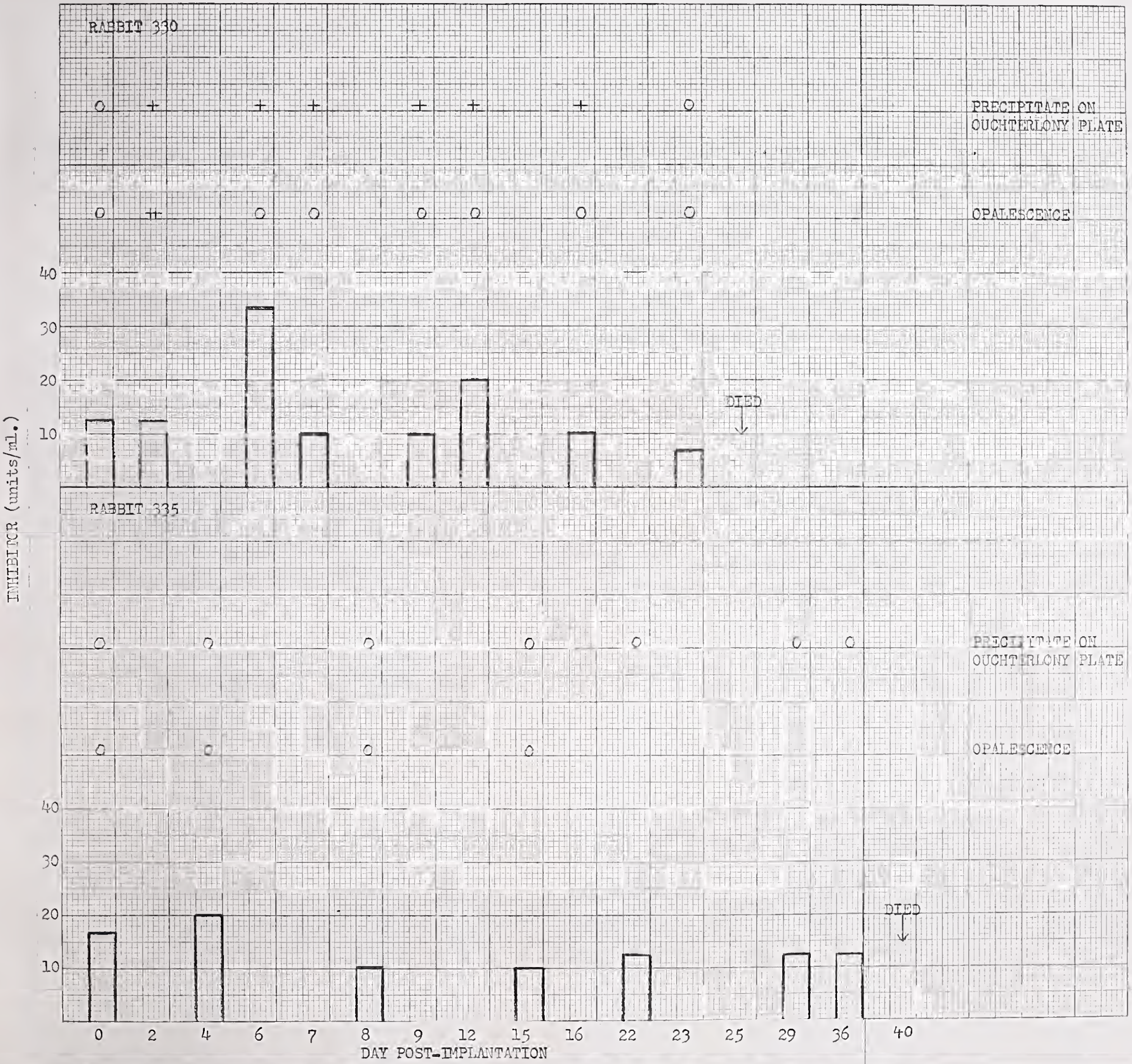

FIGURE 13- Continued.

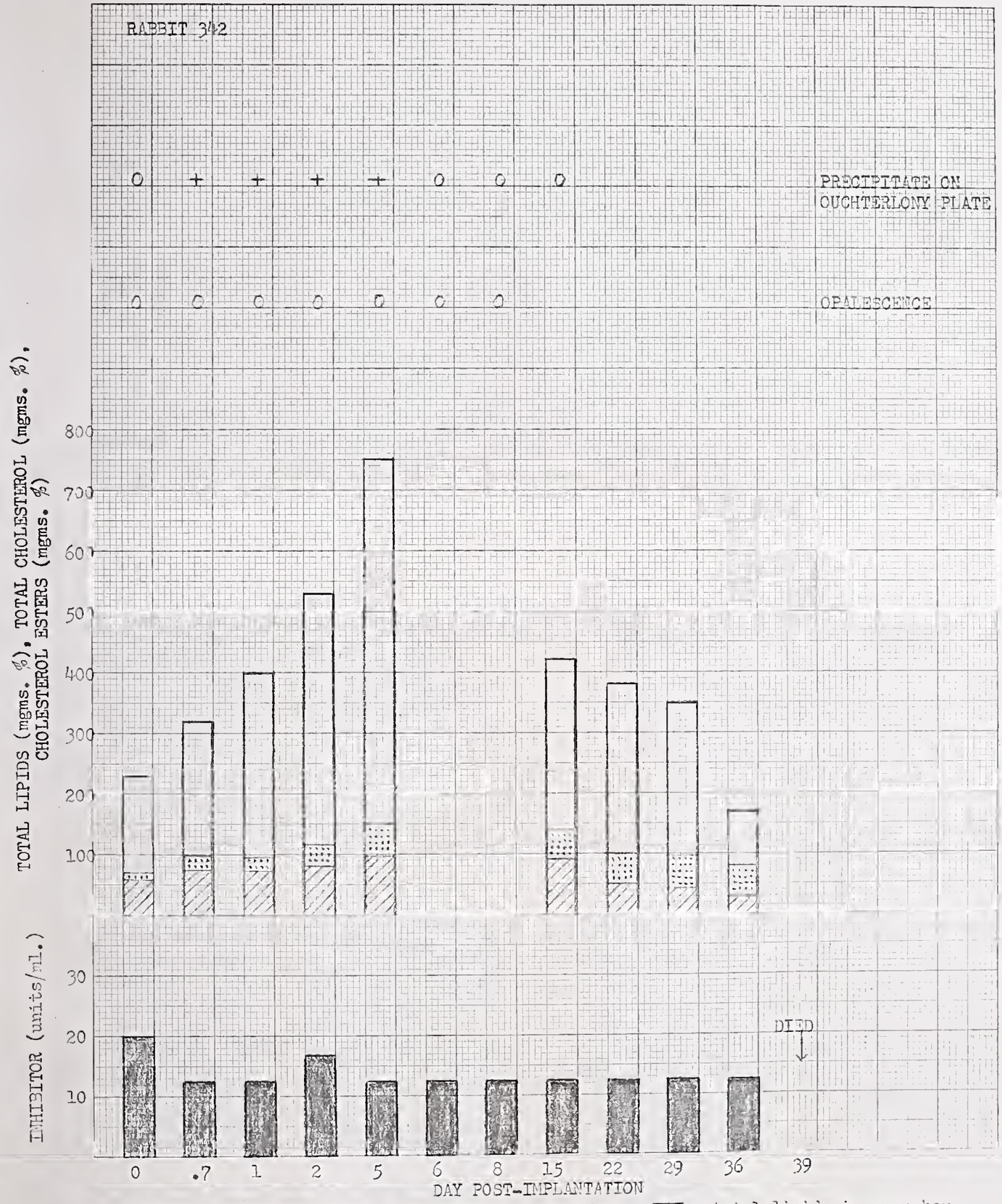

FIGURE 14- Serum inhibitor is shown in closed bar - ▓▓ ; total lipids in open bar - ▭ ; total cholesterol in stippled bar - ⁘ ; cholesterol esters in cross-hatched bar, ▨ ; opalescence, 0 - 4+, and precipitation on Ouchterlony plate of serum against streptolysin S. 0/4. are shown above.

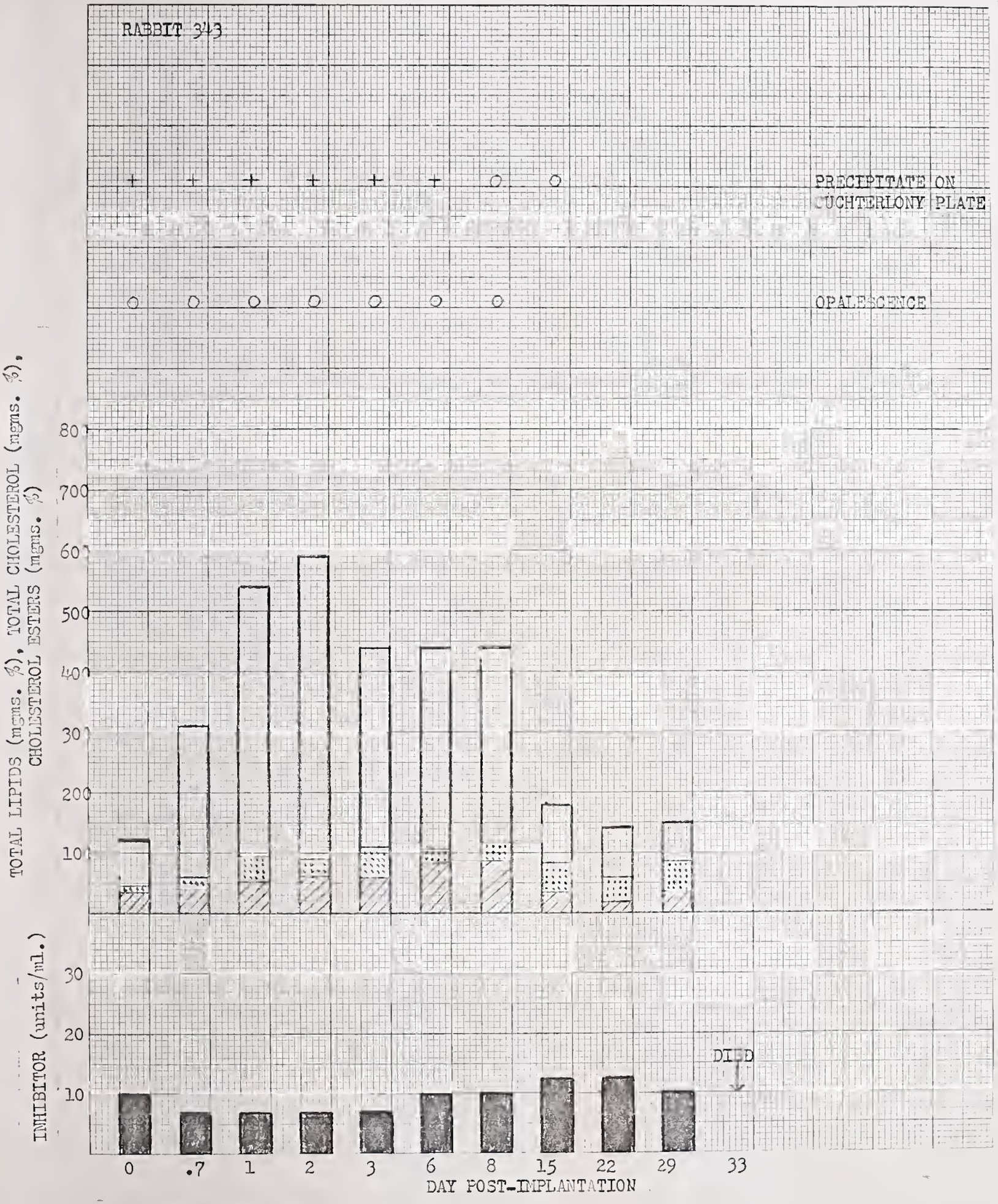

FIGURE 14- Continued.

328 and 329). The significance of this finding is not known.

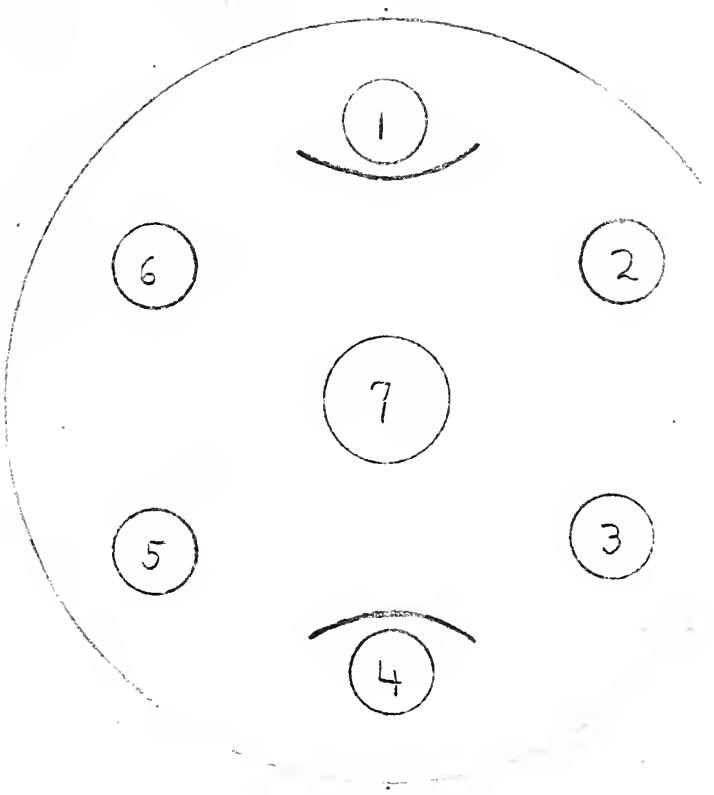

FIGURE 15--Ouchterlony plate showing the reactivity of serum (post-implantation day 2) from rabbits 328 and 330, both implanted with streptolysin S bound to a Seitz filter: 1. serum, rabbit 328; 2. RNA-se, 0.1 mgm./ml.; 3. RNA-se, 0.1 mgm./ml.; 4. serum, rabbit 330; 5. RNA core, 10 mgm./ml.; 6. RNA core, 10 mgm./ml.; 7. streptolysin S,

The following biochemical changes took place in the control rabbit (342) and in a rabbit implanted with streptolysin S bound to a Seitz filter (343) Table XI. The

post-operative observation period, which was limited by the survival of the animal, was five weeks for rabbit 342 and four weeks for rabbit 343. Total lipids, total cholesterol, cholesterol esters, and BUN went up while potassium stayed the same after the operative procedure. Total lipids returned to the pre-implantation level within five weeks for rabbit 342 and two weeks for rabbit 343. Total cholesterol returned to the pre-implantation level within five weeks for rabbit 342 and did not return to the pre-implantation level during the observation period for rabbit 343. Cholesterol esters and BUN returned to the pre-implantation level within three weeks for rabbit 342 and two weeks for rabbit 343. It is evident from Figure 14 that the transient but marked hyperlipemia observed post-implantation in one experimental (343) and one control animal (342) (the only two animals thus studied), occurred primarily in the non-cholesterol portion of the serum lipids.

The antistreptolysin O (ASO) titer was determined for all sera in the above experiment. When post-implantation sera were compared with pre-implantation sera, no elevation in the ASO titer was observed. Therefore, the lipid alterations that occurred were not such as to provoke inhibition of streptolysin O although it is known that some lipoprotein alterations may cause an inhibition of

streptolysin O.

Table XI

Rabbit	Total Lipids (mgms. %) Pre-implantation	Peak Value Post-implantation	Percent Increase
342	260	750	190%
343	120	590	390%
Rabbit	**Total Cholesterol (mgms. %) Pre-implantation**	**Peak Value Post-implantation**	**Percent Increase**
342	70	150	115%
343	45	115	155%
Rabbit	**Cholesterol Esters (mgms. %) Pre-implantation**	**Peak Value Post-implantation**	**Percent Increase**
342	58	94	62%
343	36	85	136%
Rabbit	**BUN (mgms. %) Pre-implantation**	**Peak Value Post-implantation**	**Percent Increase**
342	12	26	115%
343	21	73	250%

In the three rabbits which were followed for two weeks or more (330, 335 and 343), the absence of an ASO response argues against a streptococcal infection and also argues against the presence of streptolysin O in

the streptolysin S preparations that were injected, bound to a Seitz filter, into each rabbit.

DISCUSSION

The present data suggest that streptolysin S when injected into rabbits as a complex with MBSA is not antigenic. A precipitating antibody was obtained, but gel diffusion on Ouchterlony plates demonstrated that it was directed against RNA-se. However, conclusions on the lack of antigenicity of streptolysin S under these experimental conditions must be tempered by the knowledge that the immunization efforts were not as vigorous as they might have been because of the difficulty of obtaining streptolysin S in large amounts.

It is quite possible that immunization over a period of months might have been successful where our three-week schedule (one set of injections per week) failed. The formation of antibodies to poorly immunogenic molecules can be slow. Satisfactory levels of antibody against ACTH were found only after four to five months of immunization (14). The immunization of a large number of animals would also have been helpful, as animals may show

marked individual variations in response to a given antigen. Therefore, if one were able to obtain streptolysin S on a commercial basis it would seem worthwhile to pursue further the antigenicity of a streptolysin S - MBSA complex.

Other problems inherent in the immunization procedure may also exist. Although streptolysin S hemolytic activity could be recovered in good yield from the precipitate which formed on the addition of MBSA to streptolysin S, the streptolysin S - MBSA complex may deteriorate very shortly after injection into a rabbit. This may even occur before the complex has had an opportunity to enter and stimulate appropriate antibody-producing cells. Conversely, the streptolysin S may remain buried in the complex, unavailable to the antibody forming cells, although it was shown that rabbit serum can extract small amounts of it from the complex. Moreover, the heat-lability of streptolysin S may render it incompetent as an antigenic determinant due to heat inactivation after injection even when coupled to a carrier such as MBSA.

Another unexplained finding in the current investigation is the lack of an antibody response to methylated bovine serum albumin which is known to be antigenic under the conditions of the experiment.

The results of immunization efforts with RNA are somewhat difficult to interpret. Sera from one of the six rabbits injected with RNA, when placed on an Ouchterlony plate, showed a double precipitating system against RNA and RNA core (with a reaction of identity in each of the lines). However, it is interesting that pre-injection serum from this same rabbit showed a line of precipitation with RNA core. These results would suggest the presence of antibodies against two distinct constituents of both RNA and RNA core.

Immunization efforts with RNA core yielded in all instances (four rabbits) production of antibodies against RNA-se. No antibodies against RNA or RNA core were detected. Perhaps the most likely explanation for the lack of an antibody response against RNA and RNA core (in all of the above animals except one) is the use of too short a period of immunization; further immunization will, therefore, be attempted. Another possibility may be an inherently lower antigenicity of RNA and RNA core as compared to denatured DNA.

Immunization with streptolysin S coupled by a carbodiimide to rabbit serum albumin failed to induce an antibody response against streptolysin S. It would seem worthwhile to further pursue this approach to immunization with streptolysin S in the light of the success of

Goodfriend *et al.* with angiotensin and bradykinin (23). Since the shortcomings of this experiment are similar to those discussed for the streptolysin S - MBSA experiment, immunization of a large number of animals over a period of months might be helpful. It would also be of interest to extract the carbodiimide induced precititate in a manner similar to that used in the streptolysin S - MBSA studies to ascertain the presence of streptolysin S in the precipitate and its availability.

The experiments utilizing a Seitz filter as a "carrier" and an "adjuvant" yielded several unexpected and interesting results. In the following discussion hyperlipemia will indicate a chemically determined increase in serum lipids. In five of seven experimental animals the serum became opalescent post-implantation and in one experimental and one control animal (the only two animals thus studied) a transient but marked hyperlipemia was observed, characterized by an increase in total cholesterol and cholesterol esters. The characteristics of this hyperlipemia may be contrasted to that produced by other means. Tsaltas reported that intravenous injection of papain produced lipemia in adult rabbits (80). Further studies indicated that the lipemia was mostly due to a rise in the plasma glyceride concentration without a significant alteration in the

concentration of the other lipid fractions. The lipemia secondary to papain administration was accompanied by moderate pulmonary edema and other stress phenomena. Tsaltas and Lutton postulated that the stress reaction precipitated by the papain injection could be considered as the major cause of the lipemia (81).

It has also been reported that the injection of pituitary gland extracts produced a lipemia in rabbits which was very similar to the one produced by papain (29). Pituitary extracts were found to mobilize free fatty acid from adipose tissue. The latter was then resynthesized into triglyceride which was directly responsible for a lactescent hypertriglyceridemia.

The lipemia observed in the present study differs from those discussed above in degree as well as in composition. The percent increase in total lipids in the papain studies was about 15% as compared to a percent increase of 200% to 400% in the present study. The available chemistry determinations show a sharp increase in total cholesterol (over 100%) after the intraperitoneal implantation of the Seitz filter, whether it was impregnated with streptolysin S (experimental rabbit) or not (control rabbit). By contrast, total cholesterol did not change significantly in the papain experiment. No data are available on triglycerides for our experiments.

While cholesterol esters rose 60% to 135% in our experiments they fell 25% in the papain experiment.

In the same phase of the experiment (with respect to implantation of the Seitz filter) as was the observation of a transient hyperlipemia in the two rabbits in which it was sought, an increase in streptolysin S inhibitor (SSI) varying from 165% to 6500% was also noted in other rabbits (330, 331 and 332). This increase in SSI may be related to the marked hyperlipemia discussed above since previous studies (61) have demonstrated that the SSI activity is associated with a phospholipoprotein complex. It should be noted that two of the three rabbits showing a significant rise of SSI had been implanted with Seitz filters impregnated with a streptolysin S preparation which had not been previously Millipore-filtered (rabbits 331 and 332). Since it is known that such preparations, though clear, contain large amounts of living streptococci, it may be inferred that these rabbits developed a streptococcal infection. Unfortunately, we do not have lipid studies for these rabbits. It is pertinent to recall in this connection that Todd observed variable increases in SSI (which he termed antistreptolysin S) following prolonged courses of intravenous injections of living group A streptococci into rabbits. About half of Todd's rabbits died during

the course of the experiment with generalized streptococcal infections (78).

Another interesting observation in the immunization with streptolysin S bound to a Seitz filter was the line of precipitation which formed between most early post-implantation sera and non-dialyzed streptolysin S. This precipitation could not be due to an antibody because it occurred too soon after the implantation and because it even occurred between sera from the control rabbit (implanted intraperitoneally with a Seitz filter free of streptolysin S) and streptolysin S. Further studies will be performed to elucidate the nature of this reaction.

SUMMARY

This thesis investigated the antigenicity of streptolysin S.

1. Streptolysin S when injected into rabbits as a complex with MBSA was not antigenic. Precipitating antibodies directed against MBSA were not produced. However, a precipitating antibody was obtained which was directed against RNA-se. (RNA-se was used in the preparation of RNA core and RNA core in the preparation of streptolysin S.)

2. Streptolysin S hemolytic activity could be recovered in good yield from the precipitate which formed on the addition of MBSA to streptolysin S.
3. Post-injection serum from one of six rabbits injected with RNA had antibodies against two distinct constituents of both RNA and RNA core. Pre-injection serum from this same rabbit gave a line of precipitation with RNA core.
4. All rabbits injected with RNA core produced antibodies against RNA-se. (RNA-se was used in the preparation of RNA core.) No antibodies against RNA core were produced.
5. Streptolysin S, coupled by a carbodiimide to rabbit serum albumin, was not antigenic when injected into a rabbit.
6. Streptolysin S when passed through a Seitz filter was bound to the filter and could be eluted from it by solutions of high ionic strength.
7. Streptolysin S when bound to a Seitz filter and implanted intraperitoneally in rabbits was not antigenic. In some rabbits a marked increase in the streptolysin S inhibitor titer was observed post-implantation. In one experimental animal and one control animal (the only two animals thus studied) a transient but marked hyperlipemia was observed.

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